EXHIBIT AE

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization		(10) International Publication Number
(43) International Publication Date 8 Mar. 2012 (08.03.2012)	PCT	WO 2012/029986 A1

(51) International Patent Classification:

C12N 15/113 (2010.01) *A61P 21/04* (2006.01)

A61K 31/7125 (2006.01) *C07H 21/04* (2006.01)

(21) International Application Number: PCT/JP2011/070318

(22) International Filing Date: 31 August 2011 (31.08.2011)

(25) International Filing Language: Japanese

(26) International Publication Language: Japanese

(30) Priority Data:

Application 2010-196032 1 Sep. 2010 (01.09.2010) JP

(71) Applicant (for all designated States except US): NIPPON SHINYAKU CO., LTD. [JP/JP]; 14, Nishinosho-Monguchi-cho, Kisshoin, Minami-ku, Kyoto 601-8550 (JP). NATIONAL CENTER OF NEUROLOGY AND PSYCHIATRY [JP/JP]; 4-1-1 Ogawa-Higashi, Kodaira, Tokyo 187-8551 (JP).

(72) Inventor; and

(75) Inventor/Applicant (USA only): WATANABE, Naoki [JP/JP]; Rubio II-402, Sakura 1-chome, Tsukuba-shi, Ibaraki-ken 305-0003 (JP). SATOU, Youhei [JP/JP]; 1-13-5 Minamihibarigaoka, Takarazuka-shi, Hyogo 665-0811 (JP); TAKEDA, Shin'ichi [JP/JP]; National Center of Neurology and Psychiatry, 4-1-1 Ogawa-Higashi, Kodaira, Tokyo 187-8551 (JP). NAGATA, Tetsuya [JP/JP]; National Center of Neurology and Psychiatry, 4-1-1 Ogawa-Higashi, Kodaira, Tokyo 187-8551 (JP).

(74) Agent: KOBAYASHI, Hiroshi et al.; Abe, Ikubo & Katayama. 9F Fukuoka Building, 2-8-7 Yaesu, Chuo-ku, Tokyo 104-0028 (JP).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasia (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), Europe (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

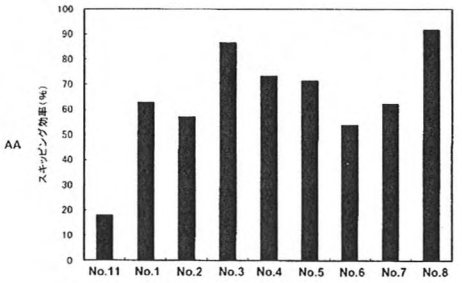
Additional Published Documents

—International Search Report (PCT Article 21 (3))

—Sequence listings presented as separate part of the description (Rule 5.2(a))

(54) **Title**: ANTISENSE NUCLEIC ACID

Figure 1



AA Skipping efficiency (%)

(57) Abstract: The object of the present invention is to offer drugs which bring about highly efficient skipping of the 53rd exon of the human dystrophin gene. The present invention offers oligomers which enable skipping of the 53rd exon of the human dystrophin gene.

SPECIFICATION

Title of the Invention

Antisense nucleic acid

Technical Field

The present invention relates to antisense oligomers which enable skipping of exon 53 of the human dystrophin gene, and to pharmaceutical compositions which include said oligomers.

Background Art

Duchenne muscular dystrophy (DMD) is the most frequent form of hereditary progressive muscle atrophy, affecting one in ca 3500 newborn males. Although motor functions are substantially unchanged from those of healthy humans in infancy, muscle weakness is observed from around 4-5 years old. Muscle weakness subsequently progresses, with inability to walk by about 12 years old, and death due to cardiac or respiratory insufficiency in the twenties; it is a serious disorder. There is currently no effective therapy for DMD, and the development of a new therapeutic agent is strongly desired.

DMD is known to be caused by a mutation in the dystrophin gene. The dystrophin gene is located on the X chromosome and is a gigantic gene consisting of DNA of 2.2 million nucleotide pairs. mRNA with 79 linked exons is synthesized by transcription from DNA to an mRNA precursor, and then removal of introns by splicing. This mRNA is translated into 3685 amino acids, to produce the dystrophin protein. The dystrophin protein contributes to the maintenance of membrane stability in muscle cells and is necessary to make muscle cells less fragile. Because the dystrophin gene from patients with DMD contains a mutation, there is hardly any expression of functional dystrophin protein in muscle cells. Therefore, in the bodies of DMD patients the structure of muscle cells cannot be maintained, and a large quantity of calcium ions flows into the muscle cells. As a result, an inflammation-like

response occurs, promoting fibrosis so that the muscle cells cannot readily be regenerated.

Becker muscular dystrophy (BMD) is also caused by a mutation in the dystrophin gene, but although the condition presents muscle weakness due to muscle atrophy, it is generally milder than DMD and muscle weakness also progresses more slowly; and in many cases, onset is in adulthood. It is thought that the differences in clinical symptoms between DMD and BMD depend on whether the reading frame for amino acids when dystrophin mRNA is translated into dystrophin protein is disrupted due to the mutation or is maintained (Non-Patent Document 1). Thus, in DMD there is hardly any expression of functional dystrophin protein because there is a mutation which shifts the amino acid reading frame; but in BMD, although some exons are lost due to the mutation, incomplete but functional dystrophin protein is produced, because the amino acid reading frame is maintained.

Exon skipping offers expectations as a method for treating DMD. This method restores the amino acid reading frame of dystrophin mRNA by modifying splicing, and induces expression of dystrophin protein with partially restored function (Non-Patent Document 2). The partial amino acid sequence which is a target for exon skipping is lost. Consequently, dystrophin protein expressed by this treatment is shorter than the normal protein, but because the amino acid reading frame is maintained, it partially retains the function of stabilizing muscle cells. Therefore, it is expected that exon skipping will give DMD which presents similar symptoms to the milder BMD. The exon skipping approach has passed through animal experiments using mice and dogs, and clinical trials on human DMD patients are in progress.

Exon skipping can be induced by binding of antisense nucleic acid targeting either the 5′ or 3′ splice site or both, or an exon-internal sequence. An exon will only be included in the mRNA when both splice sites are recognized by the spliceosome complex. Therefore, exon skipping can be induced by targeting a splice site with antisense nucleic acid. Binding of an SR protein to an exon splicing enhancer (ESE) is also thought to be necessary for recognition of an exon in the

splicing mechanism, so that exon skipping can also be induced by targeting the ESE.

Because mutations in the dystrophin gene differ among DMD patients, antisense nucleic acid suited to the site or type of gene mutation is needed. So far, antisense nucleic acids that induce exon skipping have been produced by Steve Wilton *et al.* of the University of Western Australia for all 79 exons (Non-Patent Document 3); and antisense nucleic acids which induce exon skipping have been produced by Annemieke Aartsma-Rus *et al.* in the Netherlands for 39 exons (Non-Patent Document 4).

It is thought that about 8% of all DMD patients could be treated by skipping the 53rd exon (hereinafter referred to as "exon 53"). In recent years, several research organizations have reported studies on exon 53 of the dystrophin gene as a target for exon skipping (Patent Documents 1-4; Non-Patent Document 5). However, a technique for highly efficient skipping of exon 53 has yet to be established.

Patent Document 1: WO 2006/000057 A1
Patent Document 2: WO 2004/048570 A1
Patent Document 3: US 2010/0168212 A1
Patent Document 4: WO 2010/048586 A1
Non-Patent Document 1: Monaco A. P. *et al.*, Genomics 1988; 2: p. 90-95
Non-Patent Document 2: Matsuo M., Brain Dev 1996; 18: p.167-172
Non-Patent Document 3: Wilton S. D., *et al.*, Molecular Therapy 2007 ; 15: p.1288-96
Non-Patent Document 4: Annemieke Aartsma-Rus *et al.*, (2002) Neuromuscular Disorders 12: S71-S77
Non-Patent Document 5: Linda J. Popplewell *et al.*, (2010) Neuromuscular Disorders, vol. 20, no. 2, p. 102-10

Disclosure of the Invention

Given the situation described above, an antisense oligomer that strongly induces skipping of exon 53 of the dystrophin gene, and therapeutic agents for muscular dystrophy which include such an oligomer are desired.

As a result of detailed studies of the structure of the dystrophin gene, the present inventors have found that skipping of exon 53 can be induced with high efficiency by targeting the sequence comprising peripheral nucleotides 32-56 from the 5' end of exon 53 in the mRNA precursor (hereinafter referred to as "pre-mRNA") of the dystrophin gene, by using an antisense oligomer. The present inventors have perfected the present invention based on this insight.

Thus, the present invention is as follows.

[1] An antisense oligomer, which is an antisense oligomer which enables skipping of exon 53 of the human dystrophin gene, comprising a nucleotide sequence complementary to any one of the sequences comprising nucleotides 31-53, 31-54, 31-55, 31-56, 31-57, 31-58, 32-53, 32-54, 32-55, 32-56, 32-57, 32-58, 33-53, 33-54, 33-55, 33-56, 33-57, 33-58, 34-53, 34-54, 34-55, 34-56, 34-57, 34-58, 35-53, 35-54, 35-55, 35-56, 35-57, 35-58, 36-53, 36-54, 36-55, 36-56, 36-57 or 36-58 from the 5' end of exon 53 of the human dystrophin gene.

[2] An antisense oligomer according to [1] above, which is an oligonucleotide.

[3] An antisense oligomer according to [2] above, wherein the sugar moiety and/or the phosphate bond of at least one nucleotide constituting the oligonucleotide is modified.

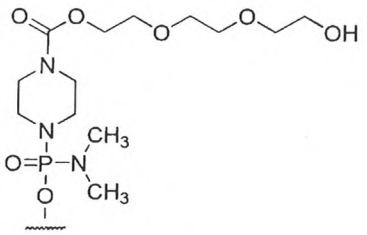
[4] An antisense oligomer according to [3] above, wherein the sugar moiety of at least one nucleotide constituting the oligonucleotide is ribose in which the 2'-OH group is replaced by any group selected from a set comprising OR, R, R'OR, SH, SR, NH_2, NHR, NR_2, N_3, CN, F, Cl, Br and I.
(Where R indicates an alkyl or aryl and R' indicates an alkylene).

[5] An antisense oligomer according to [3] or [4] above, wherein the phosphate bond of at least one nucleotide constituting the oligonucleotide is any one selected from a set comprising a phosphorothioate bond, a phosphorodithioate bond, an alkylphosphonate bond, a phosphoroamidate bond and a boranophosphate bond.

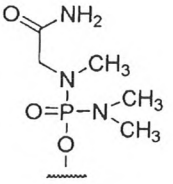
[6] An antisense oligomer according to [1] above, which is a morpholino oligomer.

[7] An antisense oligomer according to [6] above which is a phosphoroamidate morpholino oligomer.

[8] An antisense oligomer according to any one of [1]-[7] above, wherein the 5' end is any one of the groups in chemical formulae (1) to (3) below:



(1)



(2)

(3)

[9] An antisense oligomer according to any one of [1]-[8] above, comprising a nucleotide sequence complementary to a sequence comprising nucleotides 32-56 or 36-56 from the 5' end of exon 53 of the human dystrophin gene.

[10] An antisense oligomer according to any one of [1]-[8] above, comprising any one nucleotide sequence selected from a set comprising SEQ ID NO: 2-37.

[11] An antisense oligomer according to any one of [1]-[8] above, comprising any one nucleotide sequence selected from a set comprising SEQ ID NO: 11, 17, 23, 29 and 35.

[12] An antisense oligomer according to any one of [1]-[8] above, comprising the nucleotide sequence of either SEQ ID NO: 11 or 35.

[13] A pharmaceutical composition for treating muscular dystrophy, in which an active ingredient is an antisense oligomer according to any one of [1] to [12] above, or a pharmaceutically permissible salt or hydrate thereof.

An antisense oligomer of the present invention can induce skipping of exon 53 of the human dystrophin gene with high efficiency. In addition, the symptoms of Duchenne muscular dystrophy can be effectively alleviated by administering a pharmaceutical composition of the present invention.

Brief Description of the Drawings

Figure 1: is a graph showing the efficiency of skipping of exon 53 of the human dystrophin gene in human rhabdomyosarcoma cell line (RD cells).

Figure 2: is a graph showing the efficiency of skipping of exon 53 of the human dystrophin gene in fibroblasts from normal human tissue (TIG-119 cells), induced to differentiate into muscle cells by introducing the human myoD gene.

Figure 3: is a graph showing the efficiency of skipping of exon 53 of the human dystrophin gene in fibroblasts from a human DMD patient (5017 cells), induced to differentiate into muscle cells by introducing the human myoD gene.

Figure 4: is a graph showing the efficiency of skipping of exon 53 of the human dystrophin gene in fibroblasts from a human DMD patient (with deletion of exons 45-52), induced to differentiate into muscle cells by introducing the human myoD gene.

Figure 5: is a graph showing the efficiency of skipping of exon 53 of the human dystrophin gene in fibroblasts from a human DMD patient (with deletion of exons 48-52), induced to differentiate into muscle cells by introducing the human myoD gene.

Figure 6: is a graph showing the efficiency of skipping of exon 53 of the human dystrophin gene in fibroblasts from a human DMD patient (with deletion of exons 48-52), induced to differentiate into muscle cells by introducing the human myoD gene.

Figure 7: is a graph showing the efficiency of skipping of exon 53 of the human dystrophin gene in fibroblasts from a human DMD patient (with deletion of exons 45-52 or deletion of exons 48-52), induced to differentiate into muscle cells by introducing the human myoD gene.

Figure 8: is a graph showing the efficiency of skipping of exon 53 of the human dystrophin gene in fibroblasts from a human DMD patient (with deletion of exons 45-52), induced to differentiate into muscle cells by introducing the human myoD gene.

Figure 9: is a graph showing the efficiency of skipping of exon 53 (2'-OMe-S-RNA) of the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

Figure 10: is a graph showing the efficiency of skipping of exon 53 (2'-OMe-S-RNA) of the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

Figure 11: is a graph showing the efficiency of skipping of exon 53 (2'-OMe-S-RNA) of the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

Figure 12: is a graph showing the efficiency of skipping of exon 53 (2'-OMe-S-RNA) of the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

Figure 13: is a graph showing the efficiency of skipping of exon 53 (2,-OMe-S-RNA) of the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

Figure 14: is a graph showing the efficiency of skipping of exon 53 (2,-OMe-S-RNA) of the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

Figure 15: is a graph showing the efficiency of skipping of exon 53 (2'-OMe-S-RNA) of the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

Figure 16: is a graph showing the efficiency of skipping of exon 53 (2'-OMe-S-RNA) of the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

Figure 17: is a graph showing the efficiency of skipping of exon 53 (2'-OMe-S-RNA) of the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

Figure 18: is a graph showing the efficiency of skipping of exon 53 of the human dystrophin gene in human rhabdomyosarcoma cells (RD cells) at different oligomer concentrations.

Figure 19: is a graph showing the efficiency of skipping of exon 53 of the human dystrophin gene in human rhabdomyosarcoma cells (RD cells) at different oligomer concentrations.

Best Mode for Carrying Out the Invention

The present invention is described in detail below. The embodiments described below are presented as examples in order

to describe the present invention, and do not imply that the present invention is limited these embodiments. The present invention may be carried out in various ways provided that they do not depart from the gist of the invention.

It should be noted that all of the literature, laid-open patent applications, patent documents and other patent literature cited in the present description are included in this description for reference. The present description also embraces the contents of the description and drawings in the Japanese Patent Application (No. 2010-196032) filed 1 September 2010, which serves as a basis for claiming a right of priority.

1. Antisense oligomers

The present invention offers antisense oligomers (hereinafter referred to as "oligomers of the present invention") which enable skipping of exon 53 of the human dystrophin gene, comprising a nucleotide sequence complementary to any one of the sequences (hereinafter also referred to as "target sequences") comprising nucleotides 31-53, 31-54, 31-55, 31-56, 31-57, 31-58, 32-53, 32-54, 32-55, 32-56, 32-57, 32-58, 33-53, 33-54, 33-55, 33-56, 33-57, 33-58, 34-53, 34-54, 34-55, 34-56, 34-57, 34-58, 35-53, 35-54, 35-55, 35-56, 35-57, 35-58, 36-53, 36-54, 36-55, 36-56, 36-57, or 36-58 from the 5' end of exon 53 of the human dystrophin gene.

[Exon 53 of the human dystrophin gene]

In the present invention, the term "gene", in addition to genomic genes, also includes cDNA, mRNA precursors and mRNA. Preferably, the gene is an mRNA precursor, i.e., pre-mRNA.

In the human genome, the dystrophin gene is located at locus Xp21.2. The human dystrophin gene has a size of 3.0 Mbp and is the largest of the known human genes. However, the coding region of the dystrophin gene is a mere 14 kb, and said coding region is dispersed within the human dystrophin gene as 79 exons (Roberts, RG., *et al.*, Genomics, 16: 536-538 (1993)). Pre-mRNA, which is the transcript of the human dystrophin gene, undergoes splicing to produce mature mRNA of 14 kb. The

nucleotide sequence of the human wild-type dystrophin gene is known (GenBank Accession No. NM_004006).

The nucleotide sequence of exon 53 in the human wild-type dystrophin gene is shown in SEQ ID NO: 1.

The oligomers of the present invention are created in order to modify protein encoded by a DMD dystrophin gene into a BMD dystrophin protein by skipping of exon 53 of the human dystrophin gene. Therefore, exon 53 of the dystrophin gene, which is the target of exon skipping by an oligomer of the present invention, includes mutant forms as well as the wild type.

Specifically, mutant exon 53 of human dystrophin genes is a polynucleotide described in (a) or (b) below.

(a) A polynucleotide that hybridizes under stringent conditions with a polynucleotide comprising a nucleotide sequence complementary to the nucleotide sequence of SEQ ID NO:1;

(b) a polynucleotide comprising a nucleotide sequence having at least 90% homology with the nucleotide sequence of SEQ ID NO: 1.

In the present description, "polynucleotide" means DNA or RNA.

In the present description, the term "polynucleotide that hybridizes under stringent conditions" means, for example, a polynucleotide obtained by colony hybridization, plaque hybridization or Southern hybridization, etc., using as a probe all or part of a polynucleotide consisting of a nucleotide sequence complementary to the nucleotide sequence of SEQ ID NO: 1, for example. As the hybridization method, a method described, for example, in "Sambrook & Russell, Molecular Cloning: A Laboratory Manual Vol. 3, Cold Spring Harbor, Laboratory Press 2001" or "Ausubel, Current Protocols in Molecular Biology, John Wiley & Sons 1987-1997" etc., can be employed.

In this description, the term "complementary nucleotide sequence" is not limited to nucleotide sequences that form

Watson-Crick pairs with the nucleotide sequence in question, and includes nucleotide sequences which form wobble base pairs. In this connection, Watson-Crick pair are base pairs in which hydrogen bonds are formed between adenine-thymine, adenine-uracil and guanine-cytosine; and Wobble base pairs are base pairs in which hydrogen bonds are formed between guanine-uracil, inosine-uracil, inosine-adenine and inosine-cytosine. In addition, a "complementary nucleotide sequence" does not have to have 100% complementarity with the target nucleotide sequence, and can include, for example, 1-3, 1-2, or 1 non-complementary nucleotide.

In this description, the term "stringent conditions" can be any of conditions of low stringency, moderately stringent conditions or highly stringent conditions. "Conditions of low stringency" are, for example, conditions of 5×SSC, 5×Denhardts solution, 0.5% SDS and 50% formamide, at 32°C. "Moderately stringent conditions" are, for example, conditions of 5×SSC, 5×Denhardts solution, 0.5% SDS and 50% formamide, at 42°C, or 5×SSC, 1% SDS, 50 mM Tris-HCI (pH 7.5) and 50% formamide, at 42°C. "Highly stringent conditions" are, for example, 5×SSC, 5×Denhardts solution, 0.5% SDS and 50% formamide, at 50°C or 0.2×SSC and 0.1% SDS, at 65°C. Under these conditions, polynucleotides with high homology can be expected to be obtained more efficiently at higher temperatures. However, multiple factors, such as temperature, probe concentration, probe length, ionic strength, time and salt concentration, can be expected to affect hybridization stringency, and those skilled in the art can achieve similar stringency by appropriate selection of these factors.

It should be noted that when using a commercially available kit for hybridization, the Alkphos Direct Labeling and Detection System (GE Healthcare), for example, can be used. In this case, hybridized polynucleotides can be detected after incubation overnight with the labeled probe, and then washing the membrane with a primary wash buffer containing 0.1% (w/v) SDS at 55°C, in accordance with the protocol included in the kit. Alternatively, when creating a probe based on an entire

or partial nucleotide sequence complementary to the nucleotide sequence of SEQ ID NO: 1, if the probe is labeled with digoxigenin (DIG) using a commercially available reagent (for example, PCR Labeling Mix (Roche Diagnostics), etc.), hybridization can be detected by using a DIG Nucleic Acid Detection Kit (Roche Diagnostics).

Polynucleotides other than the hybridizable polynucleotides described above include polynucleotides having ≥90%, ≥91%, ≥92%, ≥93%, ≥94%, ≥95%, ≥96%, ≥97%, ≥98%, ≥99%, ≥99.1%, ≥99.2%, ≥99.3%, ≥99.4%, ≥99.5%, ≥99.6%, ≥99.7%, ≥99.8% or ≥99.9% homology with the polynucleotide of SEQ ID NO: 1, as calculated by homology search software BLAST, using the default parameters.

Homology between nucleotide sequences can be determined using algorithm BLAST (Basic Local Alignment Search Tool) by Karlin and Altschul (Proc. Natl. Acad. Sci. USA 872264-2268, 1990; Proc. Natl. Acad. Sci. USA 90: 5873, 1993). BLASTN and BLASTX programs have been developed based on the BLAST algorithm (Altschul SF, et al: J. Mol. Biol. 215: 403, 1990). When a nucleotide sequence is analysed by using BLASTN, the parameters should be, for example, score = 100 and wordlength = 12. When BLAST and Gapped BLAST programs are used, the default parameters for each program are used.

Examples of nucleotide sequences complementary to sequences comprising nucleotides 31-53, 31-54, 31-55, 31-56, 31-57, 31-58, 32-53, 32-54, 32-55, 32-56, 32-57, 32-58, 33-53, 33-54, 33-55, 33-56, 33-57, 33-58, 34-53, 34-54, 34-55, 34-56, 34-57, 34-58, 35-53, 35-54, 35-55, 35-56, 35-57, 35-58, 36-53, 36-54, 36-55, 36-56, 36-57 and 36-58 from 5' of exon 53, are shown in the table below.

Table 1

Target sequence in exon 53	Complementary nucleotide sequence	SEQ ID NO:
31-53	5'-CCGGTTCTGAAGGTGTTCTTGTA-3'	SEQ ID NO: 2
31-54	5'-TCCGGTTCTGAAGGTGTTCTTGTA-3'	SEQ ID NO: 3

31-55	5'-CTCCGGTTCTGAAGGTGTTCTTGTA-3'	SEQ ID NO: 4
31-56	5'-CCTCCGGTTCTGAAGGTGTTCTTGTA-3'	SEQ ID NO: 5
31-57	5'-GCCTCCGGTTCTGAAGGTGTTCTTGTA-3'	SEQ ID NO: 6
31-58	5'-TGCCTCCGGTTCTGAAGGTGTTCTTGTA-3'	SEQ ID NO: 7
32-53	5'-CCGGTTCTGAAGGTGTTCTTGT-3'	SEQ ID NO: 8
32-54	5'-TCCGGTTCTGAAGGTGTTCTTGT-3'	SEQ ID NO: 9
32-55	5'-CTCCGGTTCTGAAGGTGTTCTTGT-3'	SEQ ID NO:10
32-56	5'-CCTCCGGTTCTGAAGGTGTTCTTGT-3'	SEQ ID NO:11
32-57	5'-GCCTCCGGTTCTGAAGGTGTTCTTGT-3'	SEQ ID NO:12
32-58	5'-TGCCTCCGGTTCTGAAGGTGTTCTTGT-3'	SEQ ID NO:13
33-53	5'-CCGGTTCTGAAGGTGTTCTTG-3'	SEQ ID NO:14
33-54	5'-TCCGGTTCTGAAGGTGTTCTTG-3'	SEQ ID NO:15
33-55	5'-CTCCGGTTCTGAAGGTGTTCTTG-3'	SEQ ID NO:16
33-56	5'-CCTCCGGTTCTGAAGGTGTTCTTG-3'	SEQ ID NO:17
33-57	5'-GCCTCCGGTTCTGAAGGTGTTCTTG-3'	SEQ ID NO:18
33-58	5'-TGCCTCCGGTTCTGAAGGTGTTCTTG-3'	SEQ ID NO:19
34-53	5'-CCGGTTCTGAAGGTGTTCTT-3'	SEQ ID NO: 20
34-54	5'-TCCGGTTCTGAAGGTGTTCTT-3'	SEQ ID NO: 21
34-55	5'-CTCCGGTTCTGAAGGTGTTCTT-3'	SEQ ID NO: 22
34-56	5'-CCTCCGGTTCTGAAGGTGTTCTT-3'	SEQ ID NO: 23
34-57	5'-GCCTCCGGTTCTGAAGGTGTTCTT-3'	SEQ ID NO: 24
34-58	5'-TGCCTCCGGTTCTGAAGGTGTTCTT-3'	SEQ ID NO: 25
35-53	5'-CCGGTTCTGAAGGTGTTCT-3'	SEQ ID NO: 26
35-54	5'-TCCGGTTCTGAAGGTGTTCT-3'	SEQ ID NO: 27
35-55	5'-CTCCGGTTCTGAAGGTGTTCT-3'	SEQ ID NO: 28
35-56	5'-CCTCCGGTTCTGAAGGTGTTCT-3'	SEQ ID NO: 29
35-57	5'-GCCTCCGGTTCTGAAGGTGTTCT-3'	SEQ ID NO: 30
35-58	5'-TGCCTCCGGTTCTGAAGGTGTTCT-3'	SEQ ID NO: 31
36-53	5'-CCGGTTCTGAAGGTGTTC-3'	SEQ ID NO: 32
36-54	5'-TCCGGTTCTGAAGGTGTTC-3'	SEQ ID NO: 33
36-55	5'-CTCCGGTTCTGAAGGTGTTC-3'	SEQ ID NO: 34
36-56	5'-CCTCCGGTTCTGAAGGTGTTC-3'	SEQ ID NO: 35
36-57	5'-GCCTCCGGTTCTGAAGGTGTTC-3'	SEQ ID NO: 36
36-58	5'-TGCCTCCGGTTCTGAAGGTGTTC-3'	SEQ ID NO: 37

The oligomer of the present invention preferably comprises a nucleotide sequence complementary to any one of the sequences consisting of nucleotides 32-56, 33-56, 34-56, 35-56 or 36-56 from the 5' end of exon 53 of the human

dystrophin gene (for example, SEQ ID NO: 11, SEQ ID NO: 17, SEQ ID NO: 23, SEQ ID NO: 29 or SEQ ID NO: 35).

Preferably, the oligomer of the present invention comprises a nucleotide sequence complementary to either of the sequences comprising nucleotides 32-56 or 36-56 from the 5' end of exon 53 of the human dystrophin gene, (for example, SEQ ID NO: 11 or SEQ ID NO: 35).

The term "enables skipping of exon 53 of the human dystrophin gene" means that, in the case of a DMD patient with deletion of exon 52 for example, by binding an oligomer of the present invention to a site corresponding to exon 53 of the transcript (for example, pre-mRNA) of the human dystrophin gene, when said transcript is spliced the nucleotide sequence corresponding to the 5' end of exon 54 is spliced to the 3′ side of the nucleotide sequence corresponding to the 3′ end of exon 51, so that no codon frame-shift occurs and mature mRNA is formed.

Therefore, as long as an oligomer of the present invention enables skipping of exon 53 of the dystrophin gene, it does not need to have a nucleotide sequence 100% complementary to the target sequence. For example, an oligomer of the present invention may include 1-3, 1-2, or 1 nucleotide non-complementary to the target sequence.

In this connection, "binding" above means that when an oligomer of the present invention is mixed with a transcript of the human dystrophin gene, the two hybridize under physiological conditions to form double-stranded nucleic acid. Here, "under physiological conditions" means conditions of pH, salt composition and temperature adjusted to mimic the *in vivo* environment. For example, conditions of 25-40°C, and preferably 37°C, pH 5-8, and preferably pH 7.4, and sodium chloride concentration 150 mM.

Whether or not skipping of exon 53 of the human dystrophin gene is produced can be confirmed by introducing the oligomer of the present invention into dystrophin-expressing cells (for example, human rhabdomyosarcoma cells), amplifying the region surrounding exon 53 of mRNA of the human

dystrophin gene from the total RNA of the dystrophin-expressing cells by RT-PCR, and performing nested PCR or sequence analysis on said PCR-amplified product.

By recovering human dystrophin gene mRNA from the test cells, measuring the quantity of polynucleotide "A" in the band for said mRNA in which exon 53 is skipped, and the quantity of polynucleotide "B" in the bands in which exon 53 has not been skipped, skipping efficiency can be calculated from the measured values for "A" and "B" by the following equation.

$$\text{Skipping efficiency (\%)} = A/(A + B) \times 100$$

Oligomers of the present invention include, for example, oligonucleotides, morpholino oligomers, or peptide nucleic acid (PNA) oligomers, having a length of 18-28 nucleotides. A length of 21-25 nucleotides is preferred, and morpholino oligomers are preferred.

Aforementioned oligonucleotides (hereinafter referred to as "oligonucleotides of the present invention") are oligomers of the present invention having nucleotides as constituent units; these nucleotides can be ribonucleotides, deoxyribonucleotides or modified nucleotides.

A modified nucleotide is a ribonucleotide or deoxyribonucleotide in which the constituent nucleobase, sugar moiety and/or phosphate bond is/are modified.

Nucleobases include, for example, adenine, guanine, hypoxanthine, cytosine, thymine, uracil and modified such bases. Examples of modified such bases include, but are not limited to, pseudouracil, 3-methyluracil, dihydrouracil, 5-alkylcytosines (for example, 5-methylcytosine), 5-alkyluracils (for example, 5-ethyluracil), 5-halouracils (5-bromouracil), 6-azapyrimidine, 6-alkylpyrimidines (6-methyluracil), 2-thiouracil, 4-thiouracil, 4-acetylcytosine, 5-(carboxyhydroxymethyl)uracil, 5'-carboxymethylaminomethyl-2-thiouracil, 5-carboxymethylaminomethyluracil, 1-methyladenine, 1-methylhypoxanthine, 2,2-dimethylguanine, 3-methylcytosine, 2-methyladenine, 2-methylguanine, N6-methyladenine, 7-methylguanine,

5-methoxyaminomethyl-2-thiouracil, 5-methylaminomethyluracil, 5-methylcarbonylmethyluracil, 5-methyloxyuracil, 5-methyl-2-thiouracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxy-acetic acid, 2-thiocytosine, purine, 2,6-diaminopurine, 2-aminopurine, isoguanine, indole, imidazole and xanthine, etc.

Modification of the sugar moiety includes, for example, modifications of the ribose 2' position and modifications of other sites on the sugar. Modification of the ribose 2' position includes replacement of the 2'-OH of ribose by OR, R, R'OR, SH, SR, NH_2, NHR, NR_2, N_3, CN, F, Cl, Br or I. R here represents an alkyl or aryl. R' represents an alkylene.

The modifications of other sites on the sugar include, for example, replacement of O at the 4' position of ribose or deoxyribose by S, bridging between the 2' and 4' positions of the sugar, for example, LNA (locked nucleic acid) or ENA (2'-O,4'-C-ethylene-bridged nucleic acids), but are not limited to these.

Modifications of the phosphate bond includes, for example, modification by replacing the phosphodiester bond with a phosphorothioate bond, a phosphorodithioate bond, alkyl phosphonate bond, a phosphoroamidate bond or a boranophosphate bond (Enya et al: Bioorganic & Medicinal Chemistry, 2008, 18, 9154-9160) (see, for example, Japan Domestic Re-Publications of WO2006/129594 A1 and WO2006/038608 A1).

Alkyl is preferably a straight-chain or branched-chain C1-6 alkyl. Specific examples include methyl, ethyl, n-propyl, isopropyl, *n*-butyl, isobutyl, *sec*-butyl, *tert*-butyl, *n*-pentyl, isopentyl, neopentyl, *tert*-pentyl, n-hexyl and isohexyl. This alkyl may optionally be substituted; examples of applicable substituents include halogens, alkoxy groups, cyano and nitro. There may be 1-3 of these substituents.

Cycloalkyl is preferably a C5-12 cycloalkyl. Specific examples include cyclopentyl, cyclohexyl, cycloheptyl, cyclooctyl, cyclodecyl and cyclododecyl.

Halogens include fluorine, chlorine, bromine and iodine.

Alkoxy is a straight-chain or branched-chain C1-6 alkoxy, such as methoxy, ethoxy, *n*-propoxy, isopropoxy, *n*-butoxy,

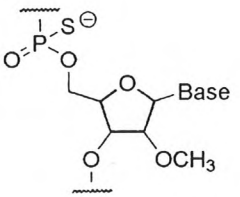
isobutoxy, *sec*-butoxy, *tert*-butoxy, *n*-pentyloxy, isopentyloxy, *n*-hexyloxy and isohexyloxy, etc. A C1-3 alkoxy is particularly preferred.

Aryl is preferably a C6-10 aryl. Specific examples include phenyl, α-naphthyl and β-naphthyl. Phenyl is particularly preferred. This aryl can be substituted; possible substituents here include, for example, alkyl, a halogen, alkoxy, cyano and nitro, and there can be 1-3 such substituents.

Alkylene is preferably a straight-chain or branched-chain C1-6 alkylene. Specific examples include methylene, ethylene, trimethylene, tetramethylene, pentamethylene, hexamethylene, 2-(ethyl)trimethylene and (methyl)tetramethylene.

Acyl includes a straight-chain or branched-chain alkanoyl or aroyl. Alkanoyl includes, for example, formyl, acetyl, 2-methylacetyl, 2,2-dimethylacetyl, propionyl, butyryl, isobutyryl, pentanoyl, 2,2-dimethylpropionyl and hexanoyl, etc. Aroyl includes, for example, benzoyl, toluoyl and naphthoyl. This aroyl can optionally be substituted at substitutable positions and can be alkyl-substituted.

Preferably, an oligonucleotide of the present invention is an oligomer of the present invention having a constituent unit represented by the general formula below, in which the 2'-OH group of ribose is methoxy-substituted and the phosphate bond is a phosphorothioate bond.

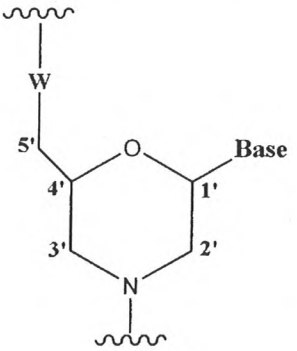


(In the formula, Base represents a nucleobase.)

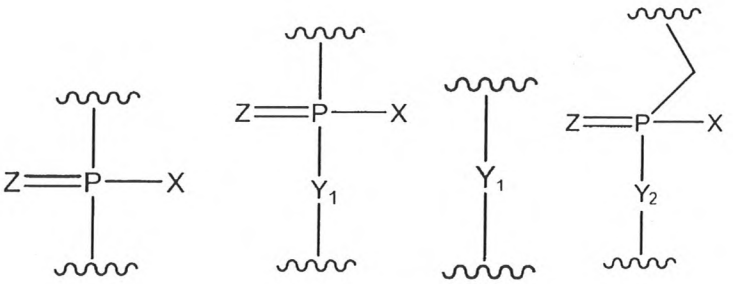
An oligonucleotide of the present invention can be synthesized easily by using different types of automated synthesizer (for example, AKTA oligopilot plus 10/100 (GE Healthcare)). Alternatively, the synthesis can also be

entrusted to a third-party organization (for example, Promega Inc., or Takara Co.), etc.

A morpholino oligomer of the present invention is an oligomer of the present invention in which a moiety represented by general formula below is a constituent unit.



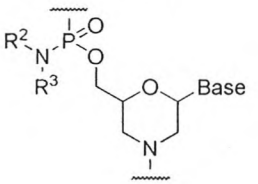
(In the formula, Base has the same significance as above;
W represents a group represented by any one of the following formulae.



(In the formulae, X represents $-CH_2R^1$, $O-CH_2R^1$, $-S-CH_2R^1$, $-NR^2R^3$ or F;
R^1 represents H or an alkyl;
R^2 and R^3, which can be the same or different, each represent H, an alkyl, a cycloalkyl or an aryl;
Y_1 represents O, S, CH_2 or NR^1;
Y_2 represents O, S or NR^1;
Z represents O or S.))

The morpholino oligomer is preferably an oligomer in which a group represented by general formula below is the

constituent unit (a phosphorodiamidate morpholino oligomer (hereinafter referred to as "PMO")).

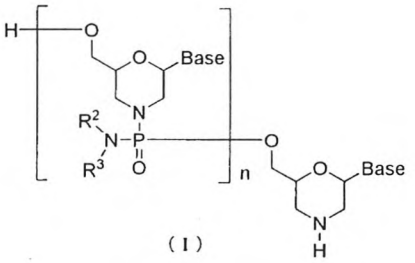


(In the formula, Base, R^2 and R^3 have the same meaning as above.)

A morpholino oligomer may be produced in accordance with WO 1991/009033 A1 or WO 2009/064471 A1, for example. In particular, a PMO can be produced by the process described in WO 2009/064471 A, or produced by the process shown below.

Process for producing PMO

In one embodiment, of PMO can be, for example, a compound represented by general formula (I) below (hereinafter referred to as PMO(I)).



(I)

[In the formula, Base, R^2 and R^3 have the same meanings as above;
n is any integer in the range 1-99, and preferably any integer in the range 18-28.]

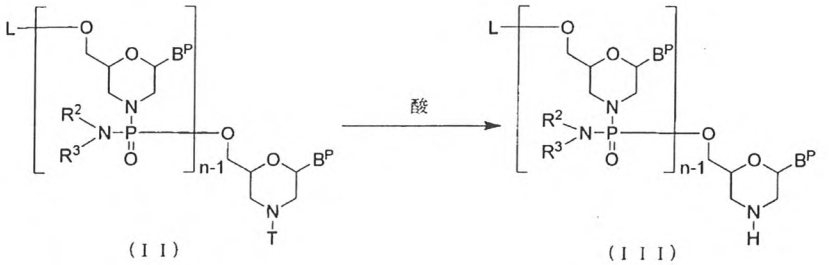
PMO(I) can be produced by a known method; for example, it can be produced by carrying out the procedures in the steps below.

There is no particular restriction as to the compounds and reagents employed in the steps below, provided that they are commonly used to prepare PMO.

In addition, all of the steps below can be carried out using a liquid-phase process or solid-phase process (using a manual or a commercially available solid-phase automated synthesizer). When PMO is produced by a solid-phase process, a process using an automated synthesizer is desirable as regards simplification of the procedure, and the accuracy of the synthesis.

(1) Step A:

A compound represented by the following general formula (II) (hereinafter referred to as Compound (II)) is reacted with an acid to prepare a compound represented by the following general formula (III) (hereinafter referred to as Compound (III)).



(II) (III)

[Key: 酸 → acid]

[In the formula, n, R2 and R3 have the same meanings as above.);

each B^P independently represents a nucleobase, which can be protected;

T represents a trityl group, a monomethoxytrityl group or a dimethoxytrityl group; and,

L represents hydrogen, an acyl or a group represented by general formula (IV) below (hereinafter referred to as group (IV).].

solid carrier---linker

(IV)

The "nucleobase" represented by B^P can be the same "nucleobase" as the "Base". The amino or hydroxy group in the nucleobase represented by B^P here can be protected.

There is no particular restriction as to such an amino group-protecting groups, provided that it is used as a

protective group for nucleic acid. Specific examples include benzoyl, 4-methoxybenzoyl, acetyl, propionyl, butyryl, isobutyryl, phenylacetyl, phenoxyacetyl, 4-*tert*-butylphenoxyacetyl, 4-isopropylphenoxyacetyl and (dimethylamino)methylene. Hydroxyl group-protecting groups include, for example, 2-cyanoethyl, 4-nitrophenethyl, phenylsulfonylethyl, methylsulfonylethyl and trimethylsilylethyl, phenyl optionally substituted at any substitutable position(s) with 1-5 electron-withdrawing group(s), diphenylcarbamoyl, dimethylcarbamoyl, diethylcarbamoyl, methylphenylcarbamoyl, 1-pyrolidinylcarbamoyl, morpholinocarbamoyl, 4-(*tert*-butylcarboxy)benzyl, 4-[(dimethylamino)carboxy]benzyl and 4-(phenylcarboxy)benzyl, (see, for example, WO 2009/064471 A).

There are no particular restrictions as to the "solid carrier", provided that it is a carrier which can be employed for solid-phase reactions of nucleic acid; however, for example, a solid carrier which (i) is sparingly soluble in reagents which may be employed in the synthesis of morpholino nucleic acid derivatives (for example, dichloromethane, acetonitrile, tetrazole, *N*-methylimidazole, pyridine, acetic anhydride, lutidine and trifluoroacetic acid), (ii) is chemically stable to reagents which may be employed in the synthesis of morpholino nucleic acid derivatives, (iii) can be chemically modified, (iv) can be loaded with the desired morpholino nucleic acid derivative, (v) is strong enough to withstand the high pressure during treatment, and (vi) has a uniform particle diameter range and distribution, is desirable. Specifically, swellable polystyrene (for example, Aminomethyl Polystyrene Resin crosslinked with 1% dibenzylbenzene (200-400 mesh) (2.4-3.0 mmol/g) (Tokyo Chemical Industry), Aminomethylated Polystyrene Resin HCI [dibenzylbenzene 1%, 100-200 mesh] (Peptide Institute, Inc.)), non-swellable polystyrene (for example, Primer Support (GE Healthcare)), PEG-grafted polystyrene (for example, NH_2-PEG resin (Watanabe Chemical Co.), TentaGel resin), controlled pore glass (controlled pore glass; CPG) (manufactured, for example, by CPG), oxalylated controlled pore glass (see, for example, Alul *et al.*, Nucleic Acids Research, Vol. 19, 1527 (1991)), a TentaGel support/amino(polyethylene glycol)-derivatized

support (see, for example, Wright *et al.*, Tetrahedron Letters, Vol. 34, 3373 (1993)), and a Poros-polystyrene/divinylbenzene copolymer.

As the "linker", a known linker conventionally used to link nucleic acids or morpholino nucleic acid derivatives can be used; examples include 3-aminopropyl, succinyl, 2,2',-diethanolsulfonyl and a long-chain alkylamino (LCAA).

This step can be carried out by the action of an acid on compound (II).

The "acid" which can be employed in this step can be trifluoroacetic acid, dichloroacetic acid or trichloroacetic acid, for example. A quantity of acid in the range of 0.1 mol equiv. to 1000 mol equiv. to 1 mol of compound (II), for example, can be suitably employed; and it is preferably in the range of 1 mol equiv. to 100 mol equiv. to 1 mol of compound (II).

An organic amine can also be used together with an aforementioned acid. Although there are no particular restrictions as to the organic amine, triethylamine can be cited as an example. A quantity of organic amine in the range of 0.01 mol equiv. to 10 mol equiv. to 1 mol of acid, for example, can be suitably employed, and it is preferably in the range of 0.1 mol equiv. to 2 mol equiv.

When a salt or mixture of an acid and an organic amine is employed in this step, this can be, for example, a salt or mixture of trifluoroacetic acid and triethylamine; more specifically, it can be a mixture of triethylamine 1 equiv. to trifluoroacetic acid 2 equiv.

The acid which can be used in this step may also be used in the form of a dilution with an appropriate solvent to give a concentration in the range of 0.1% to 30%. There are no particular restrictions as to the solvent, provided that it does not contribute to the reaction, but examples include dichloromethane, acetonitrile, an alcohol (ethanol, isopropanol or trifluoroethanol, etc.), water, or a mixture thereof.

The reaction temperature in the reaction described above is, for example, preferably in the range of 10°C to 50°C, more preferably in the range of 20°C to 40°C, and even more preferably in the range of 25°C to 35°C.

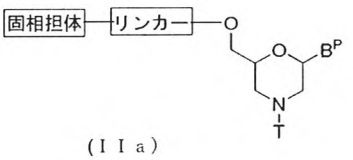
The reaction time will vary depending upon the acid and the reaction temperature employed, but a time in the range of 0.1 minute to 24 hours is normally suitable, and it is preferably in the range of 1 minute to 5 hours.

After completion of this step, if necessary a base can be added to neutralize the acid present in the system. There is no particular restriction as to the "base" but diisopropylamine can be given as an example. The base may also be employed diluted with an appropriate solvent to give a concentration in the range of 0.1% (v/v) to 30% (v/v).

There are no particular restrictions as to the solvent used in this step, provided that it does not contribute to the reaction, but examples include includes dichloromethane, acetonitrile, an alcohol (ethanol, isopropanol, trifluoroethanol, etc.) and water, and mixtures thereof. The reaction temperature is, for example, preferably in the range of 10°C to 50°C, more preferably, in the range of 20°C to 40°C, and even more preferably, in the range of 25°C to 35°C.

The reaction time will vary depending upon the base and the reaction temperature employed, but a time in the range of 0.1 minute to 24 hours is normally suitable, and it is preferably in the range of 1 minute to 5 hours.

It should be noted that a compound of general formula (IIa) below (referred to hereinafter as Compound (IIa)), in which n in Compound (II) = 1 and L is a group (IV), can be produced by the process below.

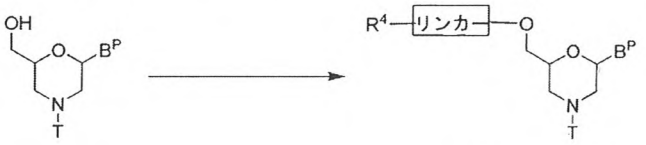


(IIa)

[In the equation B^P, T, linker (リンカー) and solid carrier (固相担体) have the same meanings as above.)].

Step 1:

A compound represented by general formula (V) below is subjected to an acylating agent to prepare a compound represented by general formula (VI) below (hereinafter referred to as Compound (VI)).

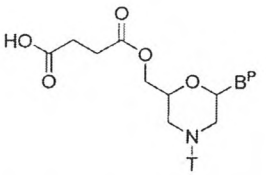


(V) (VI)

[In the formula, BP, T and linker (リンカー) have the same meanings as above;
R^4 represents a hydroxyl group, a halogen or amino.]

This step can be carried out starting from Compound (V), using a known reaction for introducing a linker.

In particular, a compound represented by general formula (VIa) below can be produced by carrying out a known esterification reaction using Compound (V) and succinic anhydride.

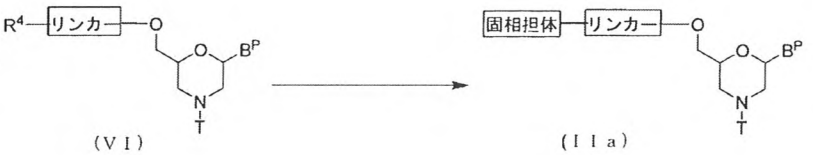


(VIa)

[In the formula, B^P and T have the same meanings as above.]

Step 2:

A step in which Compound (VI) is reacted with a solid carrier by the action of a condensing agent, to produce Compound (IIa).

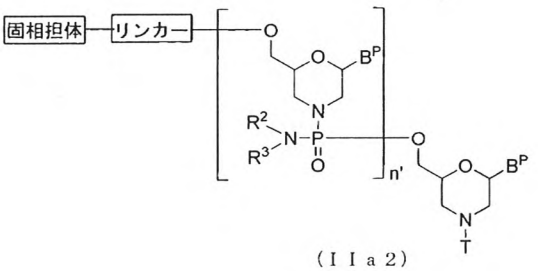


(VI) (IIa)

[In the formula, B^P, R^4, T, linker (リンカー) and solid carrier (固相担体) have the same meanings as above.]

This step can be performed by a known condensation reaction using Compound (VI) and a solid carrier.

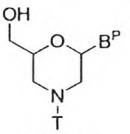
A compound represented by general formula (IIa2) below, in which n in Compound (II) is 2-99 and L is a group (IV) can be produced, starting from a Compound (IIa2), by repeating step A and step B of the PMO production process described in this description, for a desired number of times.



(IIa2)

[In the formula, B^P, R^2, R^3, T, linker (リンカー) and solid carrier (固相担体) have the same meanings as above;
n' represents 1 to 98.]

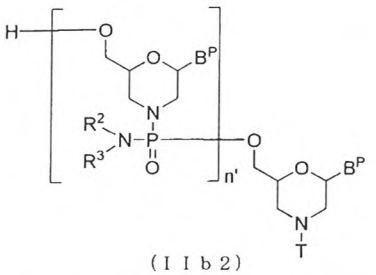
A compound of general formula (IIb) below wherein n in Compound (II) is 1 and L is hydrogen can be produced, for example, by the process described in WO1991/009033 A1.



(IIb)

[In the formula, B^P and T have the same meanings as above.]

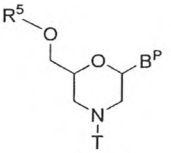
A compound represented by general formula (IIb2) below in which n in Compound (II) is 2-99 and L is hydrogen, can be produced starting from Compound (IIb), by repeating step A and step B of the PMO production process described in this description, for a desired number of times.



(IIb2)

[In the formula, B^P, n', R^2, R^3 and T have the same meanings as above.]

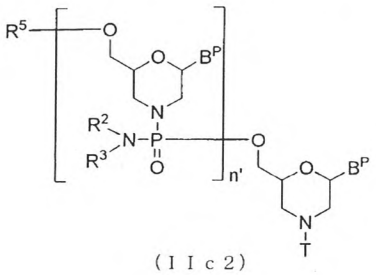
A compound represented by general formula (IIc) below wherein n in Compound (II), is 1 and L is an acyl can be produced by performing a known acylation reaction process, using Compound (IIb).



(IIc)

[In the formula, B^P and T have the same meanings as above; R^5 represents an acyl.]

A compound represented by general formula (IIc2) below in which n in Compound (II) is 2-99 and L is an acyl can be produced starting from Compound (IIc) by repeating step A and step B of the PMO production process described in this description the desired number of times.

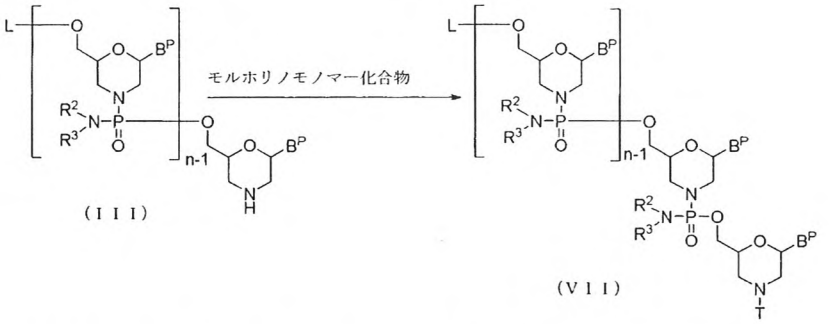


(IIc2)

[In the formula, B^P, n', R^2, R^3, R^5 and T have the same meanings as above.]

(2) Step B:

A step of producing a compound represented by general formula (VII) below (hereinafter referred to as Compound (VII)) by the action of a morpholino monomer compound on Compound (III) in the presence of a base.

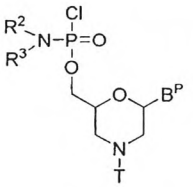


(III) (VII)

[Key: モルホリノモノマー化合物 → morpholino monomer compound]
[In the formula, B^P, L, n, R^2, R^3 and T have the same meanings as above.]

This step can be carried out by the action of a morpholino monomer compound on Compound (III) in the presence of a base.

Morpholino monomer compounds include, for example, compounds represented by general formula (VIII) below.



(VIII)

[In the formula B^P, R^2, R^3 and T are as described above.]

"Bases" which can be used in this step include, for example, diisopropylamine, triethylamine and *N*-ethylmorpholine. As the quantity of base employed, for example, a quantity in the range of 1 mol equiv. to 1000 mol equiv. to 1 mol equiv. of Compound III is appropriate, and 10 mol equiv. to 100 mol equiv. is preferred.

Morpholino monomer compounds and bases which can be used in this step can also be used diluted with a suitable solvent to give a concentration of 0.1% to 30%. There is no particular

restriction as to the solvent, provided that it does not contribute to the reaction, but examples include *N*,*N*-dimethylimidazolidone, *N*-methylpiperidone, DMF, dichloromethane, acetonitrile, tetrahydrofuran, and mixtures thereof.

The reaction temperature is preferably, for example, in the range of 0°C to 100°C, and more preferably, in the range of 10°C to 50°C.

The reaction time will vary depending upon the base and reaction temperature employed, with a range of 1 minute to 48 hours being generally appropriate, and it is preferably in the range of 30 minutes to 24 hours.

Furthermore, if necessary, after completion of this step an acylating agent can be added. "Acylating agents" include, for example, acetic anhydride, acetyl chloride and phenoxyacetic anhydride. The acylating agent may also be employed diluted with a suitable solvent to give a concentration of 0.1% to 30%. There is no particular restriction as to the solvent, provided that it does not contribute to the reaction, but examples include dichloromethane, acetonitrile, an alcohol (ethanol, isopropanol, trifluoroethanol, etc.), water, and mixtures thereof.

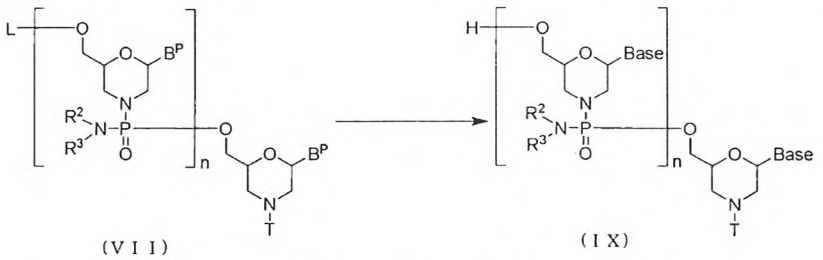
If necessary, a base such as pyridine, lutidine, collidine, triethylamine, diisopropylethylamine or *N*-ethylmorpholine, etc. can also be used together with the acylating agent. The quantity of acylating agent employed is preferably in the range of 0.1 mol equiv. to 10000 mol equiv., and more preferably in the range of 1 mol equiv. to 1000 mol equiv. As the quantity of base employed, a quantity in the range of 0.1 mol equiv. to 100 mol equiv. to 1 mol of acylating agent, for example, is appropriate, and a quantity in the range of 1 mol equiv. to 10 mol equiv. is preferred.

The reaction temperature in this reaction is preferably in the range of 10°C to 50°C, more preferably in the range of 10°C to 50°C, more preferably in the range of 20°C to 40°C, and even more preferably, in the range of 25°C to 35°C. The reaction time will vary depending upon the acylating agent and reaction temperature employed, but normally a time in the

range of 0.1 minute to 24 hours is appropriate, and preferably it is in the range of 1 minute to 5 hours.

(3) Step C:

A step in which the protecting group in Compound (VII) produced in Step B is removed by using a deprotecting agent, to prepare a compound represented by general formula (IX).



(VII) (IX)

[In the formula, Base, B^P, L, n, R^2, R^3 and T have the same meanings as above.]

This step can be performed by the action of a deprotecting agent on Compound (VII).

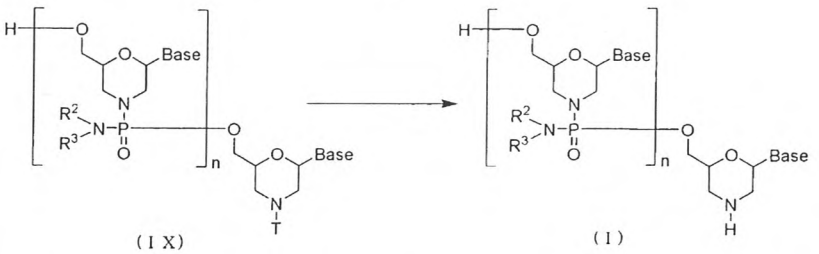
"Deprotecting agents" include, for example, concentrated ammonia water and methylamine. The "deprotecting agent" used in this step can also be used diluted, for example, with water, methanol, ethanol, isopropyl alcohol, acetonitrile, tetrahydrofuran, DMF, N,N-dimethylimidazolidone or N-methylpiperidone, or a mixture of these solvents. Of these, ethanol is preferred. As the quantity of deprotecting agent employed, for example, a quantity in the range of 1 mol equiv. to 100,000 mol equiv. to 1 mol of Compound (VII) is appropriate, and it is preferably in the range of 10 mol equiv. to 1000 mol equiv.

A reaction temperature in the range of 15°C to 75°C is appropriate, and it is preferably in the range of 40°C to 70°C, and more preferably in the range of 50°C to 60°C. The reaction time for deprotection will vary depending upon Compound (VII) and the reaction temperature, etc., but a time in the range of 10 minutes to 30 hours is appropriate; preferably it is 30

minutes to 24 hours, and more preferably it is in the range of 5 hours to 20 hours.

(4) Step D:

A step of producing PMO(I) by the action of an acid on Compound (IX) produced in step C.



(IX) (I)

[In the formula, Base, n, R^2, R^3 and T have the same meanings as above.]

This step can be carried out by adding an acid to Compound (IX).

"Acids" which can be used in this step include, for example, trichloroacetic acid, dichloroacetic acid, acetic acid, phosphoric acid and hydrochloric acid, etc. As the quantity of acid employed, use such that the pH of the solution is in the range 0.1 to 4.0 is appropriate, and use such that pH is in the range of 1.0 to 3.0 is preferred. There are no particular restrictions as regards the solvent, provided that it does not contribute to the reaction, but example include acetonitrile, water, or a mixtures of these solvents.

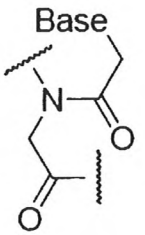
The reaction temperature is preferably in the range of 10°C to 50°C, more preferably in the range of 20°C to 40°C, and even more preferably in the range of 25°C to 35°C. The reaction time for deprotection will vary depending upon Compound (IX) and the reaction temperature, etc., but a time in the range of 0.1 minute to 5 hours is appropriate; preferably it is in the range 1 minute to 1 hour, and more preferably it is in the range of 1 minute to 30 minutes.

PMO(I) can be obtained from the reaction mixture obtained in this step by using conventional means of separation and purification, such as extraction, concentration, neutralization, filtration, centrifugation, recrystallization, reversed-phase column chromatography C_8-C_{18}, cation-exchange column chromatography, anion-exchange column chromatography, gel filtration column chromatography, high performance liquid chromatography, dialysis and/or ultrafiltration, etc., alone or in combination, to isolate and purify the desired PMO(I) (see, for example, WO 1991/09033).

When PMO(I) is purified by using reversed-phase chromatography, a mixed solution of 20mM triethylamine/acetate buffer and acetonitrile, for example, can be used as the elution solvent.

Similarly, when PMO(I) is purified using ion-exchange chromatography, a mixed solution of 1M saline and 10mM sodium hydroxide aqueous solution, for example, can be used as the elution solvent.

A peptide nucleic acid is an oligomer of the present invention in which the constituent unit is a group represented by the general formula below.



[In the formula, Base has the same meaning as above.]

Peptide nucleic acids can be prepared by referring, for example, to the following literature.

1) P. E. Nielsen, M. Egholm, R. H. Berg, O. Buchardt, Science, 254, 1497 (1991)

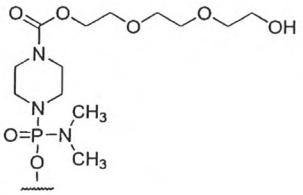
2) M. Egholm, O. Buchardt, P. E. Nielsen, R. H. Berg, Jacs., 114, 1895 (1992)

3) K. L. Dueholm, M. Egholm, C. Behrens, L. Christensen, H. F. Hansen, T. Vulpius, K. H. Petersen, R. H. Berg, P. E. Nielsen, O. Buchardt, J. Org. Chem., 59, 5767 (1994)

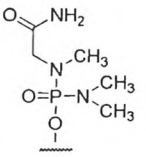
4) L. Christensen, R. Fitzpatrick, B. Gildea, K. H. Petersen, H. F. Hansen, T. Koch, M. Egholm, O. Buchardt, P. E. Nielsen, J. Coull, R. H. Berg, J. Pept. Sci., 1, 175 (1995)

5) T. Koch, H. F. Hansen, P. Andersen, T. Larsen, H. G. Batz, K. Otteson, H. Orum, J. Pept. Res., 49, 80 (1997)

In an oligomer of the present invention, the 5' can be any group in formulae (1)-(3) below. It is preferably (3) -OH.



(1)



(2)

(3)

The groups shown by (1), (2) and (3) above are referred to below as "group (1)", "group (2)" and "group (3)", respectively.

2. Pharmaceutical compositions

Oligomers of the present invention enable skipping of exon 53 with higher efficiency than antisense oligomers in the prior art. Therefore, it is predicted that administration to DMD patients of a pharmaceutical composition which includes an oligomer of the present invention will enable highly efficient alleviation of the signs and symptoms of muscular dystrophy. For example, use of a pharmaceutical composition which includes an oligomer of the present invention is economic and can decrease adverse effects, because the same therapeutic effect can be achieved even with a smaller dose than with oligomers of the prior art.

Accordingly, as another embodiment, the present invention offers pharmaceutical compositions for treating muscular dystrophy, in which an oligomer of the present invention, a pharmaceutically permissible salt or a hydrate thereof is an

active ingredient (hereinafter referred to as "compositions of the present invention").

Pharmaceutically permissible salts of an oligomer of the present invention included in a composition of the present invention include, for example, alkali metal salts such as sodium salts, potassium salts and lithium salts, alkaline earth metal salts such as calcium salts and magnesium salts, metal salts such as aluminum salts, iron salts, zinc salts, copper salts, nickel salts and cobalt salts, ammonium salts; organic amine salts such as *t*-octylamine salts, dibenzylamine salts, morpholine salts, glucosamine salts, phenylglycine alkyl ester salts, ethylenediamine salts, *N*-methylglucamine salts, guanidine salts, diethylamine salts, triethylamine salts, dicyclohexylamine salts, *N,N*-dibenzylethylenediamine salts, chloroprocaine salts, procaine salts, diethanolamine salts, N-benzylphenethylamine salts, piperazine salts, tetramethylammonium salts, tris(hydroxymethyl)aminomethane salts, hydrohalide salts such as hydrofluorates, hydrochlorides, hydrobromides and hydroiodides, inorganic acid salts such as nitrates, perchlorates, sulfates and phosphates, lower alkane sulfonates such as methanesulfonates, trifluoromethanesulfonates and ethanesulfonates, arylsulfonates such as benzenesulfonates and *p*-toluenesulfonates; organic acid salts such as acetates, malates, fumarates, succinates, citrates, tartrates, oxalates and maleates, and, amino acid salts such as glycine salts, lysine salts, arginine salts, ornithine salts, glutamates and aspartates. These salts can be produced by a known process. Alternatively, oligomers of the present invention can be included in a composition of the present invention in the form of a hydrate thereof.

There is no particular restriction as to the form of administration of a composition of the present invention, provided that it is pharmaceutically permissible form of administration, and it can be selected to suit the method of treatment; however, from the point of view of ease of delivery to muscle tissues, intravenous administration, intra-arterial administration, intramuscular administration, subcutaneous

administration, oral administration, intra-tissue administration or transdermal administration, etc., is preferred. There is also no particular restriction as to the dosage forms for a composition of the present invention, but examples include different types of injection, oral preparations, drips, inhalations, ointments and lotions.

When an oligomer of the present invention is administered to a patient with muscular dystrophy, the composition of the present invention preferably includes a carrier which promotes delivery of the oligomer to muscle tissues. There is no particular restriction as to this carrier, provided that it is a pharmaceutically permissible carrier, but examples include cationic carriers such as cationic liposomes and cationic polymers, etc., or carriers using a viral envelope. Cationic liposomes include, for example, liposomes formed using 2-*O*-(2-diethylaminoethyl)carabamoyl-1,3-*O*-dioleoylglycerol and as phospholipid as essential constituents (hereinafter referred to as "liposome A"), Oligofectamine (registered trademark) (manufactured by Invitrogen Corp.), Lipofectin (registered trademark) (Invitrogen Corp.), Lipofectamine (registered trademark) (Invitrogen Corp.), Lipofectamine 2000 (registered trademark) (Invitrogen Corp.), DMRIE-C (registered trademark) (Invitrogen Corp.), GeneSilencer (registered trademark) (Gene Therapy Systems), TransMessenger (registered trademark) (QIAGEN, Inc.), TransIT TKO (registered trademark) (Mirus) and Nucleofector II (Lonza). Of these, liposome A is preferred Cationic polymers include, for example, JetSI (registered trademark) (Qbiogene, Inc.) and Jet-PEI (registered trademark) (polyethyleneimine, Qbiogene, Inc.). An example of a carrier using a viral envelope is GenomeOne (registered trademark) (HVJ-E liposome, Ishihara Sangyo). Alternatively, a pharmaceutical device described in JP 2924179 B2 and cationic carriers described in Japanese Domestic Re-Publication of WO2006/129594 A1 and WO2008/096690 A1 can also be used.

The concentration of oligomer of the present invention included in a composition of the present invention will vary depending on the carrier, etc., but a concentration in the

range of 0.1 nM to 100 μM is appropriate, with a range of 1 nM to 10 μM being preferred, and a range of 10 nM to 1 μM being more preferred. The ratio by weight of oligomer of the present invention and carrier in contained in a composition of the present invention (carrier/oligomer of the present invention) will vary depending on the nature of the oligomer and the type of the carrier, etc., but a ratio in the range of 0.1-100 is appropriate, with a range of 1-50 being preferred, and a range of 10-20 being more preferred.

In addition to an oligomer of the present invention and a carrier described above, a composition of the present invention can also optionally include pharmaceutically permissible additives. Such additives include, for example, emulsification aids (for example, C6-22 fatty acids and pharmaceutically permissible salts thereof, albumin and dextran), stabilizers (for example, cholesterol and phosphatidic acid), isotonic agents (for example, sodium chloride, glucose, maltose, lactose, sucrose and trehalose), and pH-regulating agents (for example, hydrochloric acid, sulfuric acid, phosphoric acid, acetic acid, sodium hydroxide, potassium hydroxide and triethanolamine). One or more of these additives can be employed. As the content of these additive in a composition of the present invention ≤90 wt% is appropriate, and it is preferably ≤70 wt%, and more preferably ≤50 wt%.

A composition of the present invention can be prepared by adding an oligomer of the present invention to a carrier dispersion and adequately stirring the mixture. Additives may also be added at an appropriate stage, before or after adding the oligomer of the present invention. There are no particular restriction as to aqueous solvents that can be used when adding oligomer of the present invention is not particularly limited, provided that it is pharmaceutically permissible, but examples include injectable water or injectable distilled water, electrolyte solutions such as physiological saline, and sugar solutions such as glucose solution or maltose solution. A person skilled in the art can select conditions such as pH and temperature for this, as appropriate.

A composition of the present invention made into a liquid preparation or a lyophilized such preparation. A lyophilized preparation can be prepared by freeze-drying a liquid composition of the present invention by a conventional process. For example, after suitable sterilization, a composition of the present invention in the form of a liquid preparation can be dispensed in set quantities into vials, subjected to preliminary freezing for 2 hours at ca -40 to -20°C, and subjected to primary drying at ca. 0-10°C under reduced pressure, and then lyophilized by secondary drying at ca. 15-25°C under reduced pressure. In addition, the vials are generally sparged with nitrogen, and capped, to give a lyophilized preparation of a composition of the present invention.

A lyophilized preparation of a composition of the present invention can be employed after redissolving, in general by adding a discretionary suitable solution (reconstituting liquid). The reconstituting liquid here can be injectable water, physiological saline or another common carrier liquid. There is no particular restriction as to the volume of the reconstituting liquid, which will vary depending on the intended use, etc., but 0.5-2-times the volume prior to lyophilization, or ≤500 mL, is appropriate.

When administering a composition of the present invention, the dose is preferably adjusted, taking into account the type and dosage form of the oligomer of the present invention, the characteristics of the patient, such as age and bodyweight, the route of administration, and the nature and severity of the disease; however, the daily dose as the quantity of the oligomer of the present invention is generally in the range of 0.1 mg to 10 g/body, and preferably 1 mg to 1 g/body. These values may vary depending on type of target disease, route of administration and target molecule. Therefore, in some cases a dose lower than this range may be sufficient, and conversely, a dose higher than the range may sometimes be necessary.

Administration can be once to several times daily, or at intervals of one to several days.

As a separate embodiment of a composition of the present invention, a pharmaceutical composition which includes a vector capable of expressing an oligonucleotide of the present invention, and a carrier described above. This expression vector can be a vector capable of expressing a plurality of oligonucleotides of the present invention. As in the case of a composition of the present invention containing an oligomer of the present invention, such a composition can include pharmaceutically permissible additives. The concentration of expression vector included in the composition will vary depending upon the carrier, etc., but a concentration in the range of 0.1nM to 100μM is appropriate, a concentration in the range of 1nM to 10 μM is preferred, and a concentration in the range of 10nM to 1μM is more preferred. The ratio by weight of the expression vector and the carrier contained in the composition (carrier/expression vector) will vary depending on the nature of the expression vector and the carrier, etc., but a ratio in the range of 0.1 to 100 is appropriate, a range of 1 to 50 is preferred, and a range of 10 to 20 is more preferred. The content of the carrier included in the composition is the same as in the case of a composition of the present invention containing an oligomer of the present invention; and the method of preparation, etc., is also the same as for composition of the present invention.

The present invention is described in more detail below citing practical examples thereof and experimental examples below; however, the present invention is not limited to the description in the examples.

[Examples]

Reference Example 1]

4-{[(2S,6R)-6-(4-Benzamido-2-oxopyrimidin-1-yl)-4-trityl-morpholin-2-yl]-methoxy}-4-oxobutanoic acid supported on aminomethyl polystyrene resin

Step 1: Production of 4-{[(2S,6R)-6-(4-benzamido-2-oxo-pyrimidin-1(2H)-yl)-4-tritylmorpholin-2-yl]methoxy}-4-oxo-butanoic acid

Under an argon atmosphere, 22.0 g of N-{1-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-2-oxo-1,2-dihydro-pyrimidin-4-yl}benzamide and 7.04 g of 4-dimethylaminopyridine (4-DMAP) were suspended in 269 mL of dichloromethane, and 5.76 g of succinic anhydride was added to the suspension, with stirring at room temperature for 3 hours. 40 mL of methanol was added to the reaction solution, which was concentrated under decreased pressure. The residue was extracted using ethyl acetate and 0.5M aqueous potassium dihydrogen phosphate solution. The resulting organic layer was washed with 0.5M aqueous potassium dihydrogen phosphate solution, water and brine in this order. The resulting organic layer was dried over sodium sulfate and concentrated under decreased pressure to give 25.9 g of the intended product.

Step 2: Production of 4-{[(2S,6R)-6-(4-benzamido-2-oxo-pyrimidin-1-yl)-4-tritylmorpholin-2-yl]methoxy}-4-oxobutanoic acid supported on aminomethyl polystyrene resin

23.5 g of 4-{[(2S,6R)-6-(4-benzamido-2-oxopyrimidin-1(2H)-yl)-4-tritylmorpholin-2-yl]methoxy}-4-oxobutanoic acid was dissolved in 336 mL of pyridine (dehydrated), and 4.28 g of 4-DMAP and 40.3 g of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride were added to the solution. Then, 25.0 g of aminomethyl polystyrene resin crosslinked with 1% DVB (Tokyo Chemical Industry Co., Ltd., A1543) and 24 mL of triethylamine were added, followed by shaking at room temperature for 4 days. After the reaction, the resin was collected by filtration. The resulting resin was washed with pyridine, methanol and dichloromethane in this order, and dried under decreased pressure. 150 mL of tetrahydrofuran (dehydrated), 15 mL of acetic anhydride and 15 mL of 2,6-lutidine were added to the resulting resin, and the mixture was shaken at room temperature for 2 hours. The resin was collected by filtration, washed with pyridine, methanol and dichloromethane in this order, and dried under decreased pressure, to give 33.7 g of the intended product.

The quantity of this intended product loaded was determined by measuring the molar quantity of trityl per g of resin by measuring UV absorbance at 409 nm, using a known method. The quantity loaded on the resin was 397.4 μmol/g.

UV measurement conditions
Device: U-2910 (Hitachi, Ltd.)
Solvent: methanesulfonic acid
Wavelength: 265 nm
ε Value: 45000

[Reference Example 2]

4-Oxo-4-{[(2S,6R)-6-(6-oxo-2-[2-phenoxyacetamido]-1*H*-purin-9-yl)-4-tritylmorpholin-2-yl]methoxy)butanoic acid supported on aminomethyl polystyrene resin

Step 1: Production of N^2-(phenoxyacetyl)guanosine

100 g of guanosine was dried at 80°C under decreased pressure for 24 hours. 500 mL of pyridine (dehydrated) and 500 mL of dichloromethane (dehydrated) were added, and 401 mL of chlorotrimethylsilane was added dropwise to the mixture at 0°C under an argon atmosphere, followed by stirring at room temperature for 3 hours. The mixture was again cooled over ice, and 66.3 g of phenoxyacetyl chloride was added dropwise, followed by stirring for a further 3 hours under ice cooling. 500 mL of methanol was added to the reaction solution, and after stirring overnight at room temperature, the solvent was distilled off under decreased pressure. 500 mL of methanol was added to the residue, with concentration three times under decreased pressure. 4 L of water was added to the residue, followed by stirring for one hour under ice cooling, and the precipitate was collected by filtration. This was washed with water and then with cold methanol, and dried to give 150.2 g of the intended compound (yield: 102%) (See: Org. Lett. (2004), Vol. 6, No. 15, 2555-2557).

Step 2: N-{9-[(2R,6S)-6-(hydroxymethyl)-4-morpholin-2-yl]-6-oxo-6,9-dihydro-1H-purin-2-yl}-2-phenoxyacetamide *p*-toluenesulfonate

30 g of the compound obtained in Step 1 was suspended in 480 mL of methanol, and 130 mL of 2N hydrochloric acid was added under ice cooling. Then, 56.8 g of ammonium tetraborate tetrahydrate and 16.2 g of sodium periodate were added in this order, followed by stirring at room temperature for 3 hours. The reaction solution was ice cooled and the insoluble matter was removed by filtration; this was washed with 100 mL of methanol. The filtrate and washing liquid were bulked and ice cooled. 11.52 g of 2-picoline-borane was added to the mixture and after stirring for 20 minutes, 54.6 g of *p*-toluenesulfonic acid monohydrate was slowly added, followed by stirring at 4°C overnight. The precipitate was collected by filtration, washed with 500 mL of cold methanol, and dried, to give 17.7 g of the intended compound (yield: 43.3%).

^{1}H NMR (δ, DMSO-d6): 9.9-9.2 (2H, br), 8.35 (1H, s), 7.55 (2H, m), 7.35 (2H, m), 7.10 (2H, d, J=7.82Hz), 7.00 (3H, m), 5.95 (1H, dd, J=10.64, 2.42Hz), 4.85 (2H, s), 4.00 (1H, m), 3.90-3.60 (2H,m), 3.50-3.20 (5H, m), 2.90 (1H, m), 2.25 (3H, s)

Step 3: Production of N-{9-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-6-oxo-6,9-dihydro-1H-purin-2-yl}-2-phenoxyacetamide

2.0 g of the compound obtained in Step 2 was suspended in 30 mL of dichloromethane; 13.9 g of triethylamine and 18.3 g of trityl chloride were added under ice cooling, followed by stirring at room temperature for one hour. The reaction solution was washed with saturated sodium bicarbonate aqueous solution and then with water, and dried, and the organic layer was concentrated at decreased pressure. 40 mL of 0.2M sodium citrate buffer (pH 3)/methanol (1:4 (v/v)) was stirred into the residue. Then 40 mL of water was added, with stirring for an hour under ice cooling. The product was collected by filtration, washed with cold methanol, and dried, to give 1.84 g of the intended compound (yield: 82.0%).

Step 4: Production of 4-Oxo-4-{[(2S,6R)-6-(6-oxo-2-[2-phenoxy-acetamido]-1H-purin-9-yl)-4-tritylmorpholin-2-yl]methoxy}-butanoic acid supported on aminomethyl polystyrene resin

The title compound was produced by the same process as in Reference Example 1. However instead of *N*-{1-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-2-oxo-1,2-dihydropyrimidin-4-yl}benzamide used in Step 1 of Reference Example 1, *N*-{9-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-6-oxo-6,9-dihydro-1H-purin-2-yl}-2-phenoxyacetamide was used in this step.

[Reference Example 3]

4-{[(2S,6R)-6-(5-Methyl-2,4-dioxo-3,4-dihydropyrimidin-1-yl-4-tritylmorpholin-2-yl)methoxy]-4-oxobutanoic acid supported on aminomethyl polystyrene resin

The title compound was produced by the same process as in Reference Example 1. However instead of *N*-{1-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-2-oxo-1,2-dihydropyrimidin-4-yl}benzamide used in Step 1 of Reference Example 1, 1-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-5-methylpyrimidine-2,4(1*H*,3*H*)-dione was used in this step.

[Reference Example 4]

1, 12-Dioxo-1-(4-tritylpiperazin-1-yl)-2,5,8,11-tetraoxa-15-pentadecanoic acid supported on aminomethyl polystyrene resin

The title compound was produced by the same process as in Reference Example 1. However instead of *N*-{1-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-2-oxo-1,2-dihydropyrimidin-4-yl}benzamide used in Step 1 of Reference Example 1, 2-[2-(2-hydroxyethoxy)ethoxy]ethyl 4-tritylpiperazine-1-carboxylic acid (the compound described in WO 2009/064471 A1) was used in this step.

The PMOs shown as PMO No. 1-11 and 13-16 in Table 2 were synthesized as described in Examples 1-12 and Comparative Examples 1-3 below. The PMOs synthesized were dissolved in injectable water (Otsuka Pharmaceutical Factory, Inc.). PMO No. 12 was purchased from Gene Tools, LLC.

Table 2

PMO No.	Target sequence in exon 53	Note	SEQ ID NO:
1	31-55	5′ end: group (3)	SEQ ID NO: 4
2	32-53	5′ end: group (3)	SEQ ID NO: 8
3	32-56	5' end: group (3)	SEQ ID NO: 11
4	33-54	5′ end: group (3)	SEQ ID NO: 15
5	34-58	5′ end: group (3)	SEQ ID NO: 25
6	36-53	5′ end: group (3)	SEQ ID NO: 32
7	36-55	5′ end: group (3)	SEQ ID NO: 34
8	36-56	5′ end: group (3)	SEQ ID NO: 35
9	36-57	5' end: group (3)	SEQ ID NO: 36
10	33-57	5′ end: group (3)	SEQ ID NO: 18
11	39-69	5' end of sequence H53A(+39+69) in Non-Patent Document 3 (see Table 1): group (3)	SEQ ID NO: 38
12	30-59	5' end of sequence h53A30/1 in Non-Patent Document 5 (see Table 1): group (2)	SEQ ID NO: 39
13	32-56	5′ end: group (1)	SEQ ID NO: 11
14	36-56	5′ end: group (1)	SEQ ID NO: 35
15	30-59	5' end of sequence h53A30/1 in Non-Patent Document 5 (see Table 1): group (3)	SEQ ID NO: 39
16	23-47	5' end of sequence corresponding to SEQ ID NO: 429 described in Patent Document 4: group (3)	SEQ ID NO: 47

[Example 1]

PMO No. 8

2 g (800 μmol) of 4-{[(2S,6R)-6-(4-benzamido-2-oxo-pyrimidin-1(2H)-yl)-4-tritylmorpholin-2-yl]methoxy}-4-oxo-butanoic acid supported on aminomethyl polystyrene resin (Reference Example 1), was transferred to a reaction tank; 30 mL of dichloromethane was added, followed by standing for 30 minutes. After further washing twice with 30 mL of dichloromethane, the synthesis cycle below was started. The desired morpholino monomer compound was added in each cycle to give the nucleotide sequence of the title compound.

Table 3

Step	Reagent	Volume(mL)	Time (min)
1	Deblocking solution	30	2.0
2	Deblocking solution	30	2.0
3	Deblocking solution	30	2.0
4	Deblocking solution	30	2.0
5	Deblocking solution	30	2.0

6	Deblocking solution	30	2.0
7	Neutralizing solution	30	1.5
8	Neutralizing solution	30	1.5
9	Neutralizing solution	30	1.5
10	Neutralizing solution	30	1.5
11	Neutralizing solution	30	1.5
12	Neutralizing solution	30	1.5
13	Dichloromethane	30	0.5
14	Dichloromethane	30	0.5
15	Dichloromethane	30	0.5
16	Coupling solution B	20	0.5
17	Coupling solution A	6-11	90.0
18	Dichloromethane	30	0.5
19	Dichloromethane	30	0.5
20	Dichloromethane	30	0.5
21	Capping solution	30	3.0
22	Capping solution	30	3.0
23	Dichloromethane	30	0.5
24	Dichloromethane	30	0.5
25	Dichloromethane	30	0.5

As the deblocking solution, a solution of a mixture of trifluoroacetic acid (2 equiv.) and triethylamine (1 equiv.) at 3% (v/v) in a dichloromethane solution containing 1% (v/v) ethanol and 10% (v/v) 2,2,2-trifluoroethanol was used. As the neutralizing solution, a solution of *N,N*-diisopropylethylamine at 5% (v/v) in a dichloromethane solution containing 25% (v/v) 2-propanol was used. As coupling solution A, a solution of the morpholino monomer compound at 0.15M in 1,3-dimethyl-2-imidazolidinone containing 10% (v/v) *N,N*-diisopropylethylamine was used. As coupling solution B, a solution of *N,N*-diisopropylethylamine at 10% (v/v) in 1,3-dimethyl-2-imidazolidinone was used. As the capping solution, a solution of acetic anhydride 20% (v/v) and 2,6-lutidine 30% (v/v) in dichloromethane was used.

The aminomethyl polystyrene resin supporting the PMO synthesized above was recovered from the reaction vessel and dried under decreased pressure at room temperature for at

least 2 hours. The dried PMO supported on aminomethyl polystyrene resin was put into a reaction vessel, and 200 mL of 28% ammonia water-ethanol (1/4) was added, followed by stirring for 15 hours at 55°C. The aminomethyl polystyrene resin was removed by filtration and washed with 50 mL of water-ethanol (1/4). The resulting filtrate was concentrated under decreased pressure. The residue obtained was dissolved in 100 mL of a mixed solvent of 20 mM acetic acid-triethylamine buffer (TEAA buffer) and acetonitrile (4/1), and filtered with a membrane filter. The resulting filtrate was purified by reversed-phase HPLC. The conditions employed are given below.

Table 4

Column	XTerra MS18 (Waters, diam. 50×100 mm, 1CV=200 mL)
Flow rate	60 mL/min
Column temperature	Room temperature
Solution A	20 mM TEAA buffer
Solution B	CH_3CN
Gradient	(B) conc. 20→50%/9CV

Each fraction was analyzed and the intended product was recovered in 100 mL of acetonitrile-water (1/1); 200 mL of ethanol was added, followed by concentration under decreased pressure. After further drying under decreased pressure, a white solid was obtained. The resulting solid was suspended by adding 300 mL of 10 mM aqueous phosphoric acid. 10 mL of 2M aqueous phosphoric acid was added, followed by stirring for 15 minutes. The suspension was then neutralized by adding, 15 mL of 2M aqueous sodium hydroxide. It was then made alkaline by adding a further 15 mL of 2M aqueous sodium hydroxide, and filtered with a membrane filter (0.45 μm). After washing with 100 mL of 10 mM aqueous sodium hydroxide, the intended product was obtained as an aqueous solution.

The resulting aqueous solution containing the intended product was purified using an anion-exchange resin column. The conditions used were as follows.

Table 5

Column	Source 30Q (GE Healthcare, diam. 40 × 150 mm, 1CV=200 mL)
Flow rate	80 mL/min
Column temperature	Room temperature
Solution A	10mM aqueous sodium hydroxide
Solution B	10mM aqueous sodium hydroxide, 1M aqueous sodium chloride
Gradient	(B) conc. 5→35%/15CV

Each fraction was analyzed (HPLC) and the intended product was obtained as an aqueous solution. The resulting aqueous solution was neutralized by adding 225 mL of 0.1M phosphate buffer (pH 6.0). The solution was filtered through a membrane filter (0.45 μm). It was then desalted by ultrafiltration under the conditions below.

Table 6

Filter	PELLICON2 MINI FILTER PLBC 3K Regenerated Cellulose, Screen Type C
Size	0.1 m^2

The filtrate was concentrated to give approximately 250 mL of aqueous solution. The resulting aqueous solution was filtered with a membrane filter (0.45 μm). The aqueous solution obtained was freeze-dried, to give 1.5 g of the intended compound as a white cotton-like solid.

ESI-TOF-MS calculated: 6924.82
Measured: 6923.54

[Example 2]

PMO. No. 1

The title compound was produced by the same process as in Example 1.

MALDI-TOF-MS calculated: 8291.96
Measured: 8296.24

[Example 3]

PMO. No. 2

The title compound was produced by the same process as in Example 1.

ESI-TOF-MS calculated: 7310.13
Measured: 7309.23

[Example 4]

PMO. No. 3

The title compound was produced by the same process as in Example 1.

ESI-TOF-MS calculated: 8270.94
Measured: 8270.55

[Example 5]

PMO. No. 4

The title compound was produced by the same process as in Example 1, except that 4-(((2S,6R)-6-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1 (2*H*)-yl)-4-tritylmorpholin-2-yl)methoxy)-4-oxobutanoic acid (Reference Example 3) supported on amino-methyl polystyrene resin was employed as the starting material.

ESI-TOF-MS calculated: 7310.13
Measured: 7310.17

[Example 6]

PMO. No. 5

The title compound was produced by the same process as in Example 1, except that 4-(((2S,6R)-6-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)-4-tritylmorpholin-2-yl)methoxy)-4-oxobutanoic acid supported on aminomethyl polystyrene resin (Reference Example 3) was used as the starting material.

ESI-TOF-MS calculated: 8270.94
Measured: 8270.20

[Example 7]

PMO. No. 6

The title compound was produced by the same process as in Example 1.

ESI-TOF-MS calculated: 5964.01
Measured: 5963.68

[Example 8]

PMO. No. 7

The title compound was produced by the same process as in Example 1.

ESI-TOF-MS calculated: 6609.55
Measured: 6608.85

[Example 9]

PMO. No. 9

The title compound was produced by the same process as in Example 1, except that 4-oxo-4-(((2S,6R)-6-(6-oxo-2-(2-phenoxyacetamido)-1*H*-purin-9(6*H*)-yl)-4-tritylmorpholin-2-yl)-methoxy)butanoic acid supported on aminomethyl polystyrene resin (Reference Example 2) was used as the starting material.

ESI-TOF-MS calculated: 7280.11
Measured: 7279.42

[Example 10]

PMO. No. 10

The title compound was produced by the same process as in Example 1, except that 4-oxo-4-(((2S,6R)-6-(6-oxo-2-(2-phenoxyacetamido)-1*H*-purin-9(6*H*)-yl)-4-tritylmorpholin-2-yl)methoxy)butanoic acid supported on aminomethyl polystyrene resin (Reference Example 2) was employed as the starting material.

ESI-TOF-MS calculated: 8295.95
Measured: 8295.91

[Example 11]

PMO. No. 13

The title compound was produced by the same process as in Example 1, except that 1,12-dioxo-1-(4-tritylpiperazin-1-yl)-2,5,8,11-tetraoxa-15-pentadecanoic acid supported on amino-methyl polystyrene resin (Reference Example 4) was employed as the starting material.

ESI-TOF-MS calculated: 7276.15
Measured: 7276.69

[Example 12]

PMO. No. 14

The title compound was produced by the same process as in Example 1, except that 1,12-dioxo-1-(4-tritylpiperazin-1-yl)-2,5,8,11-tetraoxa-15-pentadecanoic acid supported on amino-methyl polystyrene resin (Reference Example 4) was employed as the starting material.

ESI-TOF-MS calculated: 8622.27
Measured: 8622.29

[Comparative Example 1]

PMO. No. 11

The title compound was produced by the same process as in Example 1.

ESI-TOF-MS calculated: 10274.63
Measured: 10273.71

[Comparative Example 2]

PMO. No. 15

The title compound was produced by the same process as in Example 1.

ESI-TOF-MS calculated: 9941.33
Measured: 9940.77

[Comparative Example 3]

PMO. No. 16

The title compound was produced by the same process as in Example 1.

ESI-TOF-MS calculated: 8238.94
Measured: 8238.69

[Experimental Example 1]

In vitro assay

Oligomers of the present invention PMO No. 1-8 and antisense oligomer PMO No. 11 were introduced at 10μM into 4×10^5 RD cells (human rhabdomyosarcoma cell line) by means of a Nucleofector II (Lonza), using Amaxa Cell Line Nucleofector Kit L. The program used was T-030.

After introduction, the cells were cultured overnight in 2 mL of Eagle's minimal essential medium (EMEM) (Sigma, the same applies below) containing 10% fetal calf serum (FCS) (Invitrogen) under conditions of 37°C and 5% CO_2. The cells were washed twice with PBS (Nissui; the same applies below), and then 500 μL of ISOGEN (Nippon Gene) was added to the cells; the cells were lysed by standing at room temperature for a several minutes, and the lysate was recovered in Eppendorf tubes. Total RNA was extracted in accordance with the protocol included with ISOGEN. The concentration of total RNA extracted was determined using a NanoDrop ND-1000 (LMS).

One-Step RT-PCR was carried out on 400 ng of the extracted total RNA, using a Titan One Tube RT-PCR Kit (Roche). Reaction solutions were prepared in accordance with the protocol included in the kit. A PTC-100 (MJ Research) was used as a thermal cycler. The RT-PCR program used was as follows.

50°C, 30 min: reverse transcription
94°C, 2 min: thermal denaturation
[94°C, 10 s; 58°C, 30 s; 68 °C, 45 s] ×30 cycles: PCR amplification
68°C, 7 min: thermal deactivation of the polymerase

The nucleotide sequences of the forward primer and reverse primer used for RT-PCR are given below.

Forward primer: 5'-AGGATTTGGAACAGAGGCGTC-3' (SEQ ID NO: 40)
Reverse primer: 5'-GTCTGCCACTGGCGGAGGTC-3' (SEQ ID NO: 41)

Next, nested PCR was carried out on the products amplified by RT-PCR above, using a Taq DNA Polymerase (Roche). The PCR program used was as follows.

94°C, 2 min: thermal denaturation
[94°C, 15 s.; 58°C, 30 s; 68 °C, 45 s] ×30 cycles: PCR amplification
68°C, 7 min: thermal deactivation of the polymerase

The nucleotide sequences of the forward primer and reverse primer used for the nested PCR above are given below.

Forward primer: 5'-CATCAAGCAGAAGGCAACAA-3' (SEQ ID NO: 42)
Reverse primer: 5'-GAAGTTTCAGGGCCAAGTCA-3' (SEQ ID NO: 43)

1 μL of the products of the nested PCR reaction above was analyzed using a Bioanalyzer (Agilent Technologies, Inc.).

The quantity of polynucleotide "A" in the band with skipping of exon 53 and the quantity of polynucleotide "B" in the band without skipping of exon 53 were measured. Skipping efficiency was found from the values measured for "A" and "B," by the following equation:

$$\text{Skipping efficiency (\%)} = A/(A+B)\times 100$$

Experimental results

The results are shown in Figure 1. This experiment showed that oligomers of the present invention PMO No. 1-8 all brought about skipping of exon 53 with a markedly higher efficiency than antisense oligomer PMO No. 11. In particular, oligomers PMO No. 3 and 8 of the present invention showed an

exon skipping efficiency at least 4-times as high as antisense oligomer PMO No. 11.

[Experimental Example 2]

In vitro assay using human fibroblasts

Human myoD gene (SEQ ID NO: 44) was introduced into TIG-119 cells (fibroblasts from normal human tissue, National Institute of Biomedical Innovation) or 5017 cells (fibroblasts from a human DMD patient, Coriell Institute for Medical Research) by using ZsGreen1 coexpression retroviral vectors.

After incubation for 4-5 days, MyoD-transformed fibroblasts ZsGreen-positive by FACS analysis were recovered and inoculated into a 12-well plate to give $5\times10^4/cm^2$. The growth medium employed was 1 mL of Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM F-12) (Invitrogen Corp.) including 10% FCS and 1% Penicillin/Streptomycin (P/S) (Sigma-Aldrich, Inc.).

After 24 hours, the medium was replaced by differentiation medium (DMEM/F-12 containing 2% equine serum (Invitrogen Corp.), 1% P/S and ITS Liquid Media Supplement (Sigma, Inc.)). Incubation was continued for 12-14 days, exchanging the medium every 2-3 days, to bring about differentiation into myotube cells.

Then the differentiation medium was replaced by a differentiation medium containing 6μM Endo-Porter (Gene Tools), and the morpholino oligomer was added to give a final concentration of 10μM. After incubation for 48 hours, total RNA was extracted from the cells using TRIzol (Invitrogen Corp.). RT-PCR was performed on 50 ng of the extracted total RNA using a QIAGEN OneStep RT-PCR Kit. The reaction solution was prepared in accordance with the protocol included in the kit. An iCycler (Bio-Rad) was used as a thermal cycler. The RT-PCR program used was as follows.

50°C, 30 min: reverse transcription
95°C, 15 min: thermal denaturation
[94°C, 1 min; 60°C ,1 min; 72 °C, 1 min] ×35 cycles: PCR amplification
72°C, 7 min: thermal deactivation of the polymerase

The primers employed were hEX51F and hEX55R.

hEX51F: 5'-CGGGCTTGGACAGAACTTAC-3' (SEQ ID NO: 45)
hEx55R: 5'-TCCTTACGGGTAGCATCCTG-3' (SEQ ID NO: 46)

The products of the RT-PCR reaction above were separated by 2% agarose gel electrophoresis, and gel images were captured using GeneFlash (Syngene). The quantity of polynucleotide "A" in the band with skipping of exon 53 and the quantity of polynucleotide "B" in the band without skipping of exon 53 were measured using ImageJ (National Institutes of Health, USA). Skipping efficiency was found from the values measured for "A" and "B" by the following equation.

$$\text{Skipping efficiency (\%)} = A/(A+B)\times 100$$

Experimental results

The results are shown in Figure 2 and 3. This experiment showed that in TIG-119 cells, the oligomers of the present invention, PMO No. 3, 8 and 9 (Figure 2) all brought about skipping of exon 53 with higher efficiency than antisense oligomer PMO No. 12 (Figure 2). In particular, the oligomers of the present invention PMO No. 3 and 8 showed exon skipping efficiency at least twice as high as that of the antisense oligomer PMO No. 12 (Figure 2).

This experiment also showed that in 5017 cells the oligomers of the present invention, PMO No. 3 and 8-10 (Figure 3), all brought about skipping of exon 53 with a higher efficiency than the antisense oligomer PMO No. 12 (Figure 3). In particular, the oligomers of the present invention PMO No. 3 and 8 showed exon skipping efficiency at least seven times as high as that of the antisense oligomer PMO No. 12 (Figure 3).

[Experimental Example 3]

In vitro assay using human fibroblasts

Skin fibroblast cell lines (fibroblasts from human DMD patients (exons 45-52 or exons 48-52)) were established by

biopsy from the inside of the left upper arm of a DMD patient with deletion of exons 45-52 or a DMD patient with deletion of exons 48-52. Human myoD gene (SEQ ID NO: 44) was introduced into the fibroblast cells using a ZsGreen1 coexpression retroviral vector.

After incubation for 4 to 5 days, MyoD-transformed fibroblasts ZsGreen-positive by FACS were recovered and inoculated into a 12-well plate to give at $5\times10^4/cm^2$. The growth medium used was 1 mL of Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) (Invitrogen Corp.) containing 10% FCS and 1% Penicillin/Streptomycin (P/S) (Sigma-Aldrich, Inc.).

After 24 hours, the medium was replaced by a differentiation medium (DMEM/F-12 containing 2% equine serum (Invitrogen Corp.), 1% P/S and ITS Liquid Media Supplement (Sigma, Inc.)). Incubation was continued for 12, 14 or 20 days, exchanging the medium every 2-3 days to bring about differentiation into myotube cells.

Then the differentiation medium was replaced by a differentiation medium containing 6μM Endo-Porter (Gene Tools), and a morpholino oligomer was added thereto to give a final concentration of 10μM. After incubation for 48 hours, total RNA was extracted from the cells using TRIzol (Invitrogen Corp.). RT-PCR was performed on 50 ng of the extracted total RNA using a QIAGEN OneStep RT-PCR Kit. The reaction solution was prepared in accordance with the protocol included with the kit. An iCycler (BioRad) was used as a thermal cycler. The RT-PCR program used was as follows.

50°C, 30 min: reverse transcription
95°C, 15 min: thermal denaturation
[94°C, 1 min; 60°C, 1 min; 72 °C, 1 min] ×35 cycles: PCR amplification
72°C, 7 min: thermal deactivation of the polymerase

The primers used were hEx44F and h55R.

hEx44F: 5'-TGTTGAGAAATGGCGGCGT-3' (SEQ ID NO: 48)
hEx55R: 5'-TCCTTACGGGTAGCATCCTG-3' (SEQ ID NO: 46)

The products of the RT-PCR reaction above were separated by 2% agarose gel electrophoresis, and gel images were captured using GeneFlash (Syngene). The quantity of polynucleotide "A" in the band with skipping of exon 53 and the quantity of polynucleotide "B" in the band without skipping of exon 53 were measured using ImageJ (National Institutes of Health, USA). Skipping efficiency was found from the values measured for "A" and "B" by the following equation.

$$\text{Skipping efficiency (\%)} = A/(A+B)\times 100$$

Experimental results

The results are shown in Figures 4 and 5. This experiment showed that in cells from a DMD patient with deletion of exons 45-52 (Figure 4) or deletion of exons 48-52 (Figure 5), the oligomers of the present invention PMO No. 3 and 8 brought about skipping of exon 53 with a high efficiency of ≥80%. It also showed that in the cells from the DMD patient with deletion of exons 45-52 the oligomers of the present invention PMO No. 3 and 8 brought about skipping of exon 53 with a higher efficiency than that of the antisense oligomer PMO No. 15 (Figure 4).

[Experimental Example 4]

Western blotting

Oligomer of the present invention PMO No. 8 was added to cells at a concentration of 10μM, and proteins were extracted from the cells after 72 hours, using RIPA buffer (Thermo Fisher Scientific) containing Complete Mini (Roche Applied Science), and quantified using a BCA protein assay kit (Thermo Fisher Scientific). The proteins were subjected to electrophoresis in NuPAGE Novex 3-8% Tris-Acetate Gel (Invitrogen) at 150V for 75 minutes, and transferred onto PVDF film (Millipore) using a semi-dry blotter. The PVDF film was blocked with a 5% ECL Blocking agent (GE Healthcare) and the film was then incubated in a solution of anti-dystrophin antibody (NCL-Dys1, Novocastra). After further incubation in peroxidase-conjugated goat-antimouse IgG (Catalog No., Bio-

Rad) solution, the film was colored using ECL Plus Western blotting system (GE Healthcare).

Immunostaining

An oligomer of the present invention, PMO No. 3 or 8, was added to the cells, and after 72 hours, the cells were fixed in 3% paraformaldehyde for 10 minutes. They were incubated in 10% Triton-X for 10 minutes. After blocking with PBS containing 10% goat serum, the film was incubated anti-dystrophin antibody (NCL-Dys1, Novocastra) solution, and then incubated in antimouse IgG antibody (Invitrogen) solution. It was mounted with Pro Long Gold Antifade reagent (Invitrogen), and observed with a fluorescence microscope.

Experimental results

The results are shown in Figures 6 and 7. This experiment confirmed, by western blotting (Figure 6) and immunostaining (Figure 7), that the oligomers of the present invention PMO No. 3 and 8 induce expression of the dystrophin protein.

[Experimental Example 5]

In vitro assay using human fibroblasts

The experiment was performed as in Experimental Example 3.

Experimental results

The results are shown in Figure 8. This experiment showed that in cells from a DMD patients with deletion of exons 45-52, oligomers of the present invention PMO No. 3 to 8 brought about skipping of exon 53 with higher efficiency than oligomers of the present invention PMO No. 13 and 14 (Figure 8).

[Experimental Example 6]

In vitro assay

Experiments were carried out using the 2'-O-methoxy-phosphorothioate (2'-OMe-S-RNA) antisense oligomers described in SEQ ID NO: 49-123. The antisense oligomers used for the assay were purchased from Japan Bio Services Co., Ltd. The sequences of antisense oligomers are presented below.

Table 7

Antisense oligomer	Nucleotide sequence	SEQ NO:
H53_39-69	CAUUCAACUGUUGCCUCCGGUUCUGAAGGUG	49
H53_1-25	UCCCACUGAUUCUGAAUUCUUUCAA	50
H53_6-30	CUUCAUCCCACUGAUUCUGAAUUCU	51
H53_11-35	UUGUACUUCAUCCCACUGAUUCUGA	52
H53_16-40	UGUUCUUGUACUUCAUCCCACUGAU	53
H53_21-45	GAAGGUGUUCUUGUACUUCAUCCCA	54
H53_26-50	GUUCUGAAGGUGUUCUUGUACUUCA	55
H53_31-55	CUCCGGUUCUGAAGGUGUUCUUGUA	56
H53_36-60	GUUGCCUCCGGUUCUGAAGGUGUUC	57
H53_41-65	CAACUGUUGCCUCCGGUUCUGAAGG	58
H53_46-70	UCAUUCAACUGUUGCCUCCGGUUCU	59
H53_51-75	ACAUUUCAUUCAACUGUUGCCUCCG	60
H53_56-80	CUUUAACAUUUCAUUCAACUGUUGC	61
H53_61-85	GAAUCCUUUAACAUUUCAUUCAACU	62
H53_66-90	GUGUUGAAUCCUUUAACAUUUCAUU	63
H53_71-95	CCAUUGUGUUGAAUCCUUUAACAUU	64
H53_76-100	UCCAGCCAUUGUGUUGAAUCCUUUA	65
H53_81-105	UAGCUUCCAGCCAUUGUGUUGAAUC	66
H53_86-110	UUCCUUAGCUUCCAGCCAUUGUGUU	67
H53_91-115	GCUUCUUCCUUAGCUUCCAGCCAUU	68
H53_96-120	GCUCAGCUUCUUCCUUAGCUUCCAG	69
H53_101-125	GACCUGCUCAGCUUCUUCCUUAGCU	70
H53_106-130	CCUAAGACCUGCUCAGCUUCUUCCU	71
H53_111-135	CCUGUCCUAAGACCUGCUCAGCUUC	72
H53_116-140	UCUGGCCUGUCCUAAGACCUGCUCA	73
H53_121-145	UUGGCUCUGGCCUGUCCUAAGACCU	74
H53_126-150	CAAGCUUGGCUCUGGCCUGUCCUAA	75
H53_131-155	UGACUCAAGCUUGGCUCUGGCCUGU	76
H53_136-160	UUCCAUGACUCAAGCUUGGCUCUGG	77
H53_141-165	CCUCCUUCCAUGACUCAAGCUUGGC	78

H53_146-170	GGGACCCUCCUUCCAUGACUCAAGC	79
H53_151-175	GUAUAGGGACCCUCCUUCCAUGACU	80
H53_156-180	CUACUGUAUAGGGACCCUCCUUCCA	81
H53_161-185	UGCAUCUACUGUAUAGGGACCCUCC	82
H53_166-190	UGGAUUGCAUCUACUGUAUAGGGAC	83
H53_171-195	UCUUUUGGAUUGCAUCUACUGUAUA	84
H53_176-200	GAUUUUCUUUUGGAUUGCAUCUACU	85
H53_181-205	UCUGUGAUUUUCUUUUGGAUUGCAU	86
H53_186-210	UGGUUUCUGUGAUUUUCUUUUGGAU	87
H53_84-108	CCUUAGCUUCCAGCCAUUGUGUUGA	88
H53_88-112	UCUUCCUUAGCUUCCAGCCAUUGUG	89
H53_119-143	GGCUCUGGCCUGUCCUAAGACCUGC	90
H53_124-148	AGCUUGGCUCUGGCCUGUCCUAAGA	91
H53_128-152	CUCAAGCUUGGCUCUGGCCUGUCCU	92
H53_144-168	GACCCUCCUUCCAUGACUCAAGCUU	93
H53_149-173	AUAGGGACCCUCCUUCCAUGACUCA	94
H53_153-177	CUGUAUAGGGACCCUCCUUCCAUGA	95
H53_179-203	UGUGAUUUUCUUUUGGAUUGCAUCU	96
H53_184-208	GUUUCUGUGAUUUUCUUUUGGAUUG	97
H53_188-212	CUUGGUUUCUGUGAUUUUCUUUUGG	98
H53_29-53	CCGGUUCUGAAGGUGUUCUUGUACU	99
H53_30-54	UCCGGUUCUGAAGGUGUUCUUGUAC	100
H53_32-56	CCUCCGGUUCUGAAGGUGUUCUUGU	101
H53_33-57	GCCUCCGGUUCUGAAGGUGUUCUUG	102
H53—34-58	UGCCUCCGGUUCUGAAGGUGUUCUU	103
H53_35-59	UUGCCUCCGGUUCUGAAGGUGUUCU	104
H53_37-61	UGUUGCCUCCGGUUCUGAAGGUGUU	105
H53_38-62	CUGUUGCCUCCGGUUCUGAAGGUGU	106
H53_39-63	ACUGUUGCCUCCGGUUCUGAAGGUG	107
H53_40-64	AACUGUUGCCUCCGGUUCUGAAGGU	108
H53_32-61	UGUUGCCUCCGGUUCUGAAGGUGUUCUUGU	109
H53_32-51	GGUUCUGAAGGUGUUCUUGU	110

H53_35-54	UCCGGUUCUGAAGGUGUUCU	111
H53_37-56	CCUCCGGUUCUGAAGGUGUU	112
H53_40-59	UUGCCUCCGGUUCUGAAGGU	113
H53_42-61	UGUUGCCUCCGGUUCUGAAG	114
H53_32-49	UUCUGAAGGUGUUCUUGU	115
H53_35-52	CGGUUCUGAAGGUGUUCU	116
H53_38-55	CUCCGGUUCUGAAGGUGU	117
H53_41-58	UGCCUCCGGUUCUGAAGG	118
H53_44-61	UGUUGCCUCCGGUUCUGA	119
H53_35-49	UUCUGAAGGUGUUCU	120
H53_40-54	UCCGGUUCUGAAGGU	121
H53-45-59	UUGCCUCCGGUUCUG	122
H53_45-62	CUGUUGCCUCCGGUUCUG	123

RD cells (human rhabdomyosarcoma cell line) were inoculated at 3×10^5 into a 6-well plate and cultured overnight in 2 mL of Eagle's minimal essential medium (EMEM) (Sigma, Inc.; the same applies below) containing 10% fetal calf serum (FCS) (Invitrogen Corp.) under conditions of 37°C and 5% CO_2. Complexes of each of the aforementioned antisense oligomers for skipping of exon 53 (Japan Bio Services) (1μM) with Lipofectamine 2000 (Invitrogen Corp.) were prepared, and 200 μL aliquots were added to RD cells in 1.8 mL of fresh culture medium, to give a final concentration of 100 nM.

After addition, the cells were cultured overnight. The cells were then washed twice with PBS (Nissui, the same applies below) and then 500 μL of ISOGEN (Nippon Gene) was added to the cells; the cells were lysed after leaving them at room temperature for several minutes, and the lysate was collected in Eppendorf tubes. Total RNA was extracted in accordance with the protocol included with ISOGEN. The concentration of the total RNA extracted was measured using a NanoDrop ND-1000 (LMS).

One-Step RT-PCR was carried out on 400 ng of the extracted total RNA using a Titan One Tube RT-PCR Kit (Roche). The reaction solution was prepared in accordance with the

protocol included in the kit. A PTC-100 (MJ Research) was used as a thermal cycler. The RT-PCR program used was as follows.

50°C, 30 min: reverse transcription
94°C, 2 min: thermal denaturation
[94°C, 10 s; 58°C, 30 s; 68 °C, 45 s] ×30 cycles: PCR amplification
68°C, 7 min: thermal denaturation of the polymerase

The nucleotide sequences of the forward primer and reverse primer employed for RT-PCR were as follows.

Forward primer: 5'-CATCAAGCAGAAGGCAACAA-3' (SEQ ID NO: 42)
Reverse primer: 5'-GAAGTTTCAGGGCCAAGTCA-3' (SEQ ID NO: 43)

Nested PCR was then carried out on the amplified product of the RT-PCR above, using a Taq DNA Polymerase (Roche). The PCR program used was as follows.

94°C, 2 min: thermal denaturation
[94°C, 15 s; 58°C, 30 s; 68 °C, 45 s] ×30 cycles: PCR amplification
68°C, 7 min: thermal denaturation of the polymerase

The nucleotide sequences of the forward primer and reverse primer used for the nested PCR above were as follows.

Forward primer: 5'-AGGATTTGGAACAGAGGCGTC-3' (SEQ ID NO: 40)
Reverse primer: 5'-GTCTGCCACTGGCGGAGGTC-3' (SEQ ID NO: 41)

1 µl of the product of the nested PCR above was analyzed using a Bioanalyzer (Agilent Technologies, Inc.).

The quantity of polynucleotide "A" in the band with skipping of exon 53 and the quantity of polynucleotide "B" in the band without skipping of exon 53 were measured. Skipping efficiency was found from the values measured for "A" and "B," by the following equation.

$$\text{Skipping efficiency (\%)} = A/(A+B)\times 100$$

Experimental results

The results are shown in Figures 9-17. This experiment showed that highly efficient skipping of exon 53 is brought about by designing antisense oligomers to 31-61 from the 5' end of exon 53 of the human dystrophin gene.

[Experimental Example 7]

Antisense oligomers were introduced at 0.3-30μM into 3.5×10^5 RD cells (human rhabdomyosarcoma cell line) by means of a Nucleofector II (Lonza) using Amaxa Cell Line Nucleofector Kit L. The Program used was T-030.

After introduction, the cells were cultured overnight in 2 mL of Eagle's minimal essential medium (EMEM) (Sigma, Inc.; the same applies below) containing 10% fetal calf serum (FCS) (Invitrogen Corp.) under conditions of 37°C and 5% CO_2. The cells were washed twice with PBS (Nissui; the same applies below) and then 500 μl of ISOGEN (Nippon Gene) was added to the cells; the cells were lysed after leaving them at room temperature for several minutes, and the lysate was collected in Eppendorf tubes. Total RNA was extracted in accordance with the protocol included with ISOGEN. The concentration of the total RNA extracted was measured using a NanoDrop ND-1000 (LMS).

One-Step RT-PCR was carried out on 400 ng of the extracted total RNA using a QIAGEN OneStep RT-PCR Kit (QIAGEN Inc.). The reaction solution was prepared in accordance with the protocol included in the kit. A PTC-100 (MJ Research) was used as a thermal cycler. The RT-PCR program used was as follows.

50°C, 30 min: reverse transcription
95°C, 15 min: thermal denaturation
[94°C, 30 s; 60°C, 30 s; 72°C, 1 min] ×35 cycles: PCR amplification
72°C, 10 min: thermal denaturation of the polymerase

The nucleotide sequences of the forward primer and reverse primer used for RT-PCR were as follows.

Forward primer: 5'-CATCAAGCAGAAGGCAACAA-3' (SEQ ID NO: 42)
Reverse primer: 5'-GAAGTTTCAGGGCCAAGTCA-3' (SEQ ID NO: 43)

1 µl of the products of the PCR above above was analyzed using a Bioanalyzer (Agilent Technologies, Inc.).

The quantity of polynucleotide "A" in the band with skipping of exon 53 and the quantity of polynucleotide "B" in the band without skipping of exon 53 were measured. Skipping efficiency was found from the values measured for "A" and "B" by the following equation.

$$\text{Skipping efficiency (\%)} = A/(A+B)\times 100$$

Experimental results

The results are shown in Figures 18 and 19. This experiment showed that the oligomer of the present invention PMO No. 8 brought about skipping of exon 53 with a markedly high efficiency as compared to the antisense oligomers PMO No. 15 and 16 (Figure 18). It also showed that the oligomers of the present invention PMO No. 3 and 8 brought about skipping of exon 53 with a markedly higher efficiency than the oligomers of the present invention PMO No. 13 and 14 (Figure 19). These results indicate that, for the same sequences, sequences with a 5'-terminal -OH group show higher skipping efficiency.

Industrial Applicability

The experimental results presented in the experimental examples indicate that, in all the cellular environments, oligomers of the present invention (PMO No. 1-10) brought about skipping of exon 53 with markedly higher efficiency than oligomers of the prior art (PMO No. 11, 12, 15 and 16). The 5017 cells used in Experimental Example 2 were cells isolated from DMD patients, and the fibroblasts from DMD patients used in Experimental Examples 3 and 5 were also suitable target cells for skipping of exon 53. Particularly in Experimental Examples 3 and 5, the oligomers of the present invention showed an exon 53 skipping efficiency of ≥90% in the cells

from DMD patients that were the target for skipping of exon 53; and therefore, oligomers of the present invention can also be expected to bring about highly efficient skipping of exon 53 when actually administered to DMD patients.

Therefore, the oligomers of the present invention are very useful for the treatment of DMD.

Sequence listing free text

SEQ ID NO: 2: synthetic nucleic acid
SEQ ID NO: 3: synthetic nucleic acid
SEQ ID NO: 4: synthetic nucleic acid
SEQ ID NO: 5: synthetic nucleic acid
SEQ ID NO: 6: synthetic nucleic acid
SEQ ID NO: 7: synthetic nucleic acid
SEQ ID NO: 8: synthetic nucleic acid
SEQ ID NO: 9: synthetic nucleic acid
SEQ ID NO: 10: synthetic nucleic acid
SEQ ID NO: 11: synthetic nucleic acid
SEQ ID NO: 12: synthetic nucleic acid
SEQ ID NO: 13: synthetic nucleic acid
SEQ ID NO: 14: synthetic nucleic acid
SEQ ID NO: 15: synthetic nucleic acid
SEQ ID NO: 16: synthetic nucleic acid
SEQ ID NO: 17: synthetic nucleic acid
SEQ ID NO: 18: synthetic nucleic acid
SEQ ID NO: 19: synthetic nucleic acid
SEQ ID NO: 20: synthetic nucleic acid
SEQ ID NO: 21: synthetic nucleic acid
SEQ ID NO: 22: synthetic nucleic acid
SEQ ID NO: 23: synthetic nucleic acid
SEQ ID NO: 24: synthetic nucleic acid
SEQ ID NO: 25: synthetic nucleic acid
SEQ ID NO: 26: synthetic nucleic acid
SEQ ID NO: 27: synthetic nucleic acid
SEQ ID NO: 28: synthetic nucleic acid
SEQ ID NO: 29: synthetic nucleic acid
SEQ ID NO: 30: synthetic nucleic acid
SEQ ID NO: 31: synthetic nucleic acid
SEQ ID NO: 32: synthetic nucleic acid

SEQ ID NO: 33: synthetic nucleic acid
SEQ ID NO: 34: synthetic nucleic acid
SEQ ID NO: 35: synthetic nucleic acid
SEQ ID NO: 36: synthetic nucleic acid
SEQ ID NO: 37: synthetic nucleic acid
SEQ ID NO: 38: synthetic nucleic acid
SEQ ID NO: 39: synthetic nucleic acid
SEQ ID NO: 40: synthetic nucleic acid
SEQ ID NO: 41: synthetic nucleic acid
SEQ ID NO: 42: synthetic nucleic acid
SEQ ID NO: 43: synthetic nucleic acid
SEQ ID NO: 45: synthetic nucleic acid
SEQ ID NO: 46: synthetic nucleic acid
SEQ ID NO: 47: synthetic nucleic acid
SEQ ID NO: 48: synthetic nucleic acid
SEQ ID NO: 49: synthetic nucleic acid
SEQ ID NO: 50: synthetic nucleic acid
SEQ ID NO: 51: synthetic nucleic acid
SEQ ID NO: 52: synthetic nucleic acid
SEQ ID NO: 53: synthetic nucleic acid
SEQ ID NO: 54: synthetic nucleic acid
SEQ ID NO: 55: synthetic nucleic acid
SEQ ID NO: 56: synthetic nucleic acid
SEQ ID NO: 57: synthetic nucleic acid
SEQ ID NO: 58: synthetic nucleic acid
SEQ ID NO: 59: synthetic nucleic acid
SEQ ID NO: 60: synthetic nucleic acid
SEQ ID NO: 61: synthetic nucleic acid
SEQ ID NO: 62: synthetic nucleic acid
SEQ ID NO: 63: synthetic nucleic acid
SEQ ID NO: 64: synthetic nucleic acid
SEQ ID NO: 65: synthetic nucleic acid
SEQ ID NO: 66: synthetic nucleic acid
SEQ ID NO: 67: synthetic nucleic acid
SEQ ID NO: 68: synthetic nucleic acid
SEQ ID NO: 69: synthetic nucleic acid
SEQ ID NO: 70: synthetic nucleic acid
SEQ ID NO: 71: synthetic nucleic acid
SEQ ID NO: 72: synthetic nucleic acid

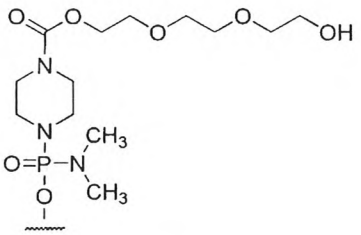
SEQ ID NO: 73: synthetic nucleic acid
SEQ ID NO: 74: synthetic nucleic acid
SEQ ID NO: 75: synthetic nucleic acid
SEQ ID NO: 76: synthetic nucleic acid
SEQ ID NO: 77: synthetic nucleic acid
SEQ ID NO: 78: synthetic nucleic acid
SEQ ID NO: 79: synthetic nucleic acid
SEQ ID NO: 80: synthetic nucleic acid
SEQ ID NO: 81: synthetic nucleic acid
SEQ ID NO: 82: synthetic nucleic acid
SEQ ID NO: 83: synthetic nucleic acid
SEQ ID NO: 84: synthetic nucleic acid
SEQ ID NO: 85: synthetic nucleic acid
SEQ ID NO: 86: synthetic nucleic acid
SEQ ID NO: 87: synthetic nucleic acid
SEQ ID NO: 88: synthetic nucleic acid
SEQ ID NO: 89: synthetic nucleic acid
SEQ ID NO: 90: synthetic nucleic acid
SEQ ID NO: 91: synthetic nucleic acid
SEQ ID NO: 92: synthetic nucleic acid
SEQ ID NO: 93: synthetic nucleic acid
SEQ ID NO: 94: synthetic nucleic acid
SEQ ID NO: 95: synthetic nucleic acid
SEQ ID NO: 96: synthetic nucleic acid
SEQ ID NO: 97: synthetic nucleic acid
SEQ ID NO: 98: synthetic nucleic acid
SEQ ID NO: 99: synthetic nucleic acid
SEQ ID NO: 100: synthetic nucleic acid
SEQ ID NO: 101: synthetic nucleic acid
SEQ ID NO: 102: synthetic nucleic acid
SEQ ID NO: 103: synthetic nucleic acid
SEQ ID NO: 104: synthetic nucleic acid
SEQ ID NO: 105: synthetic nucleic acid
SEQ ID NO: 106: synthetic nucleic acid
SEQ ID NO: 107: synthetic nucleic acid
SEQ ID NO: 108: synthetic nucleic acid
SEQ ID NO: 109: synthetic nucleic acid
SEQ ID NO: 110: synthetic nucleic acid
SEQ ID NO: 111: synthetic nucleic acid

SEQ ID NO: 112: synthetic nucleic acid
SEQ ID NO: 113: synthetic nucleic acid
SEQ ID NO: 114: synthetic nucleic acid
SEQ ID NO: 115: synthetic nucleic acid
SEQ ID NO: 116: synthetic nucleic acid
SEQ ID NO: 117: synthetic nucleic acid
SEQ ID NO: 118: synthetic nucleic acid
SEQ ID NO: 119: synthetic nucleic acid
SEQ ID NO: 120: synthetic nucleic acid
SEQ ID NO: 121: synthetic nucleic acid
SEQ ID NO: 122: synthetic nucleic acid
SEQ ID NO: 123: synthetic nucleic acid

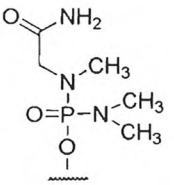
Sequence listing

CLAIMS

1. Antisense oligomer which is an antisense oligomer which enables skipping of exon 53 of the human dystrophin gene, comprising a nucleotide sequence complementary to any one of the sequences comprising nucleotides 31-53, 31-54, 31-55, 31-56, 31-57, 31-58, 32-53, 32-54, 32-55, 32-56, 32-57, 32-58, 33-53, 33-54, 33-55, 33-56, 33-57, 33-58, 34-53, 34-54, 34-55, 34-56, 34-57, 34-58, 35-53, 35-54, 35-55, 35-56, 35-57, 35-58, 36-53, 36-54, 36-55, 36-56, 36-57 or 36-58 from the 5' end of exon 53 of the human dystrophin gene.
2. Antisense oligomer according to claim 1, which is an oligonucleotide.
3. Antisense oligomer according to claim 2, wherein the sugar moiety and/or the phosphate bond of at least one nucleotide constituting the aforementioned oligonucleotide is modified.
4. Antisense oligomer according to claim 3, wherein the sugar moiety of at least one nucleotide constituting the aforementioned oligonucleotide is ribose in which the 2'-OH group is replaced by any group selected from a set comprising OR, R, R'OR, SH, SR, NH_2, NHR, NR_2, N_3, CN, F, Cl, Br and I.
(Where R indicates an alkyl or aryl and R' indicates an alkylene).
5. Antisense oligomer according to claim 3 or 4, wherein the phosphate bond of at least one nucleotide constituting the oligonucleotide is any one selected from a set comprising a phosphorothioate bond, a phosphorodithioate bond, an alkylphosphonate bond, a phosphoroamidate bond and a boranophosphate bond.
6. Antisense oligomer according to claim 1, which is a morpholino oligomer.
7. Antisense oligomer according to claim 6, which is a phosphoroamidate morpholino oligomer.
8. Antisense oligomer according to claim 6 or 7, wherein the 5' end is any one of the groups in chemical formulae (1) to (3) below:



(1)



(2)

(3)

9. Antisense oligomer according to any one of claims 1-8, comprising a nucleotide sequence complementary to a sequence comprising nucleotides 32-56 or 36-56 from the 5' end of exon 53 of the human dystrophin gene.

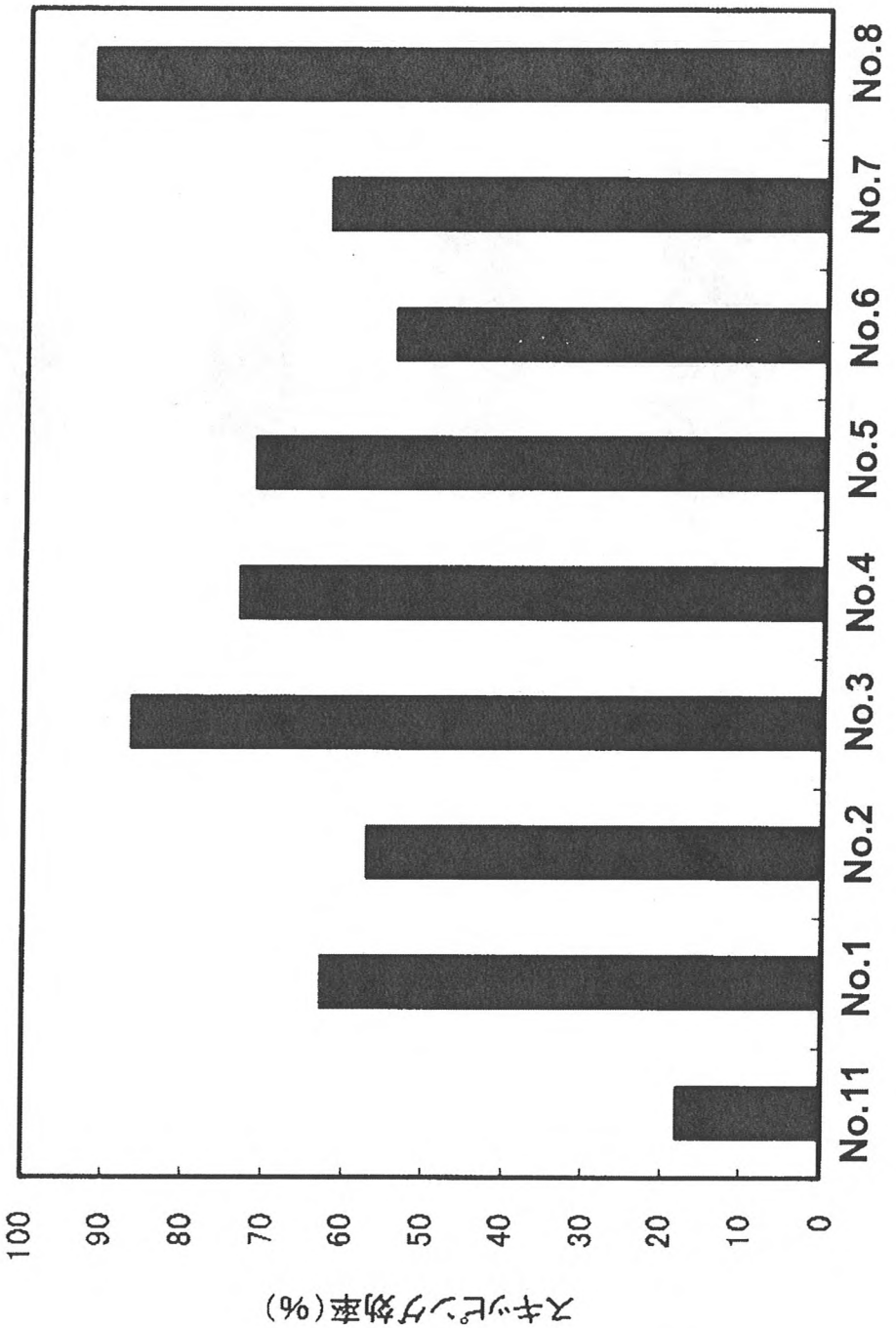
10. Antisense oligomer according to any one of claims 1-8, comprising any one nucleotide sequence selected from a set comprising SEQ ID NO: 2-37.

11. Antisense oligomer according to any one of claims 1-8, comprising any one nucleotide sequence selected from a set comprising SEQ ID NO: 11, 17, 23, 29 and 35.

12. Antisense oligomer according to any one of claims 1-8, comprising the nucleotide sequence of either SEQ ID NO: 11 or 35.

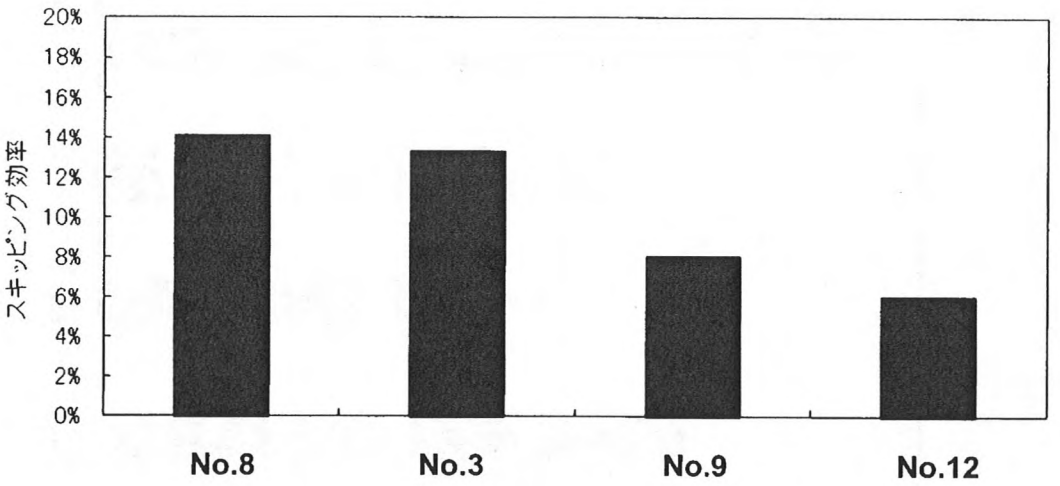
13. Pharmaceutical composition for treating muscular dystrophy, in which an active ingredient is an antisense oligomer according to any one of claims 1 to 12, or a pharmaceutically permissible salt or hydrate thereof.

Figure 1



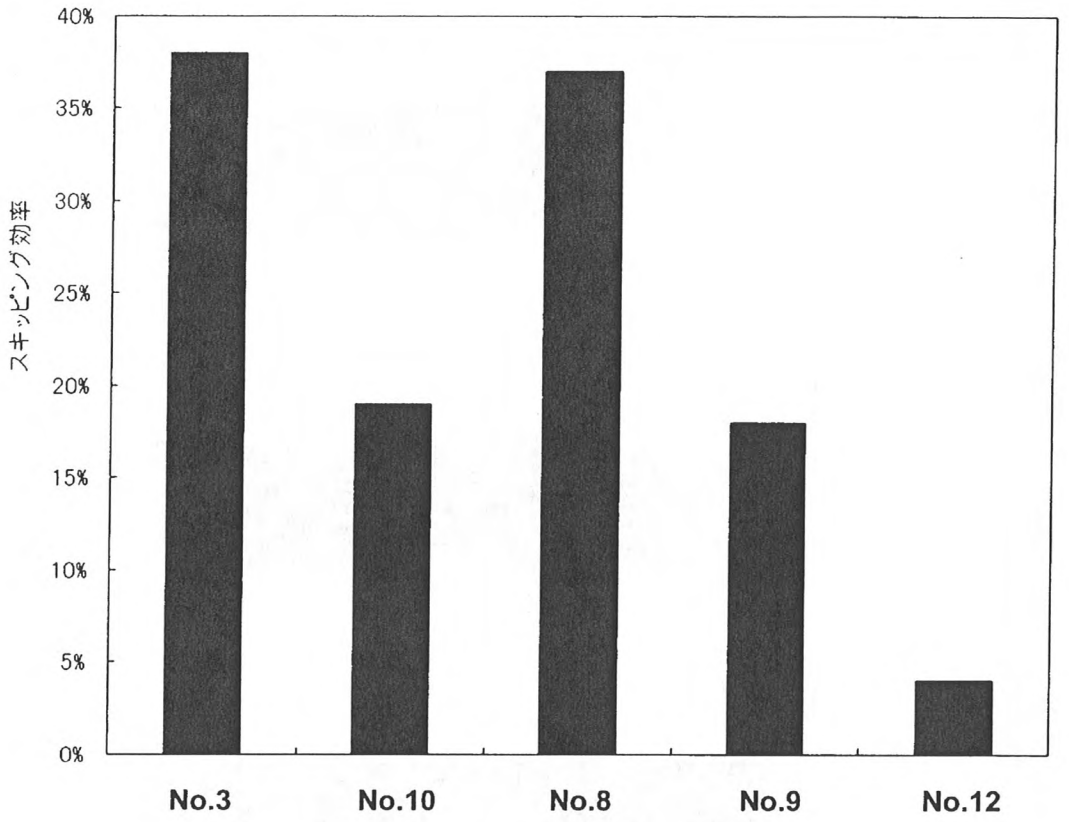
[y-axis Skipping efficiency (%)]

Figure 2



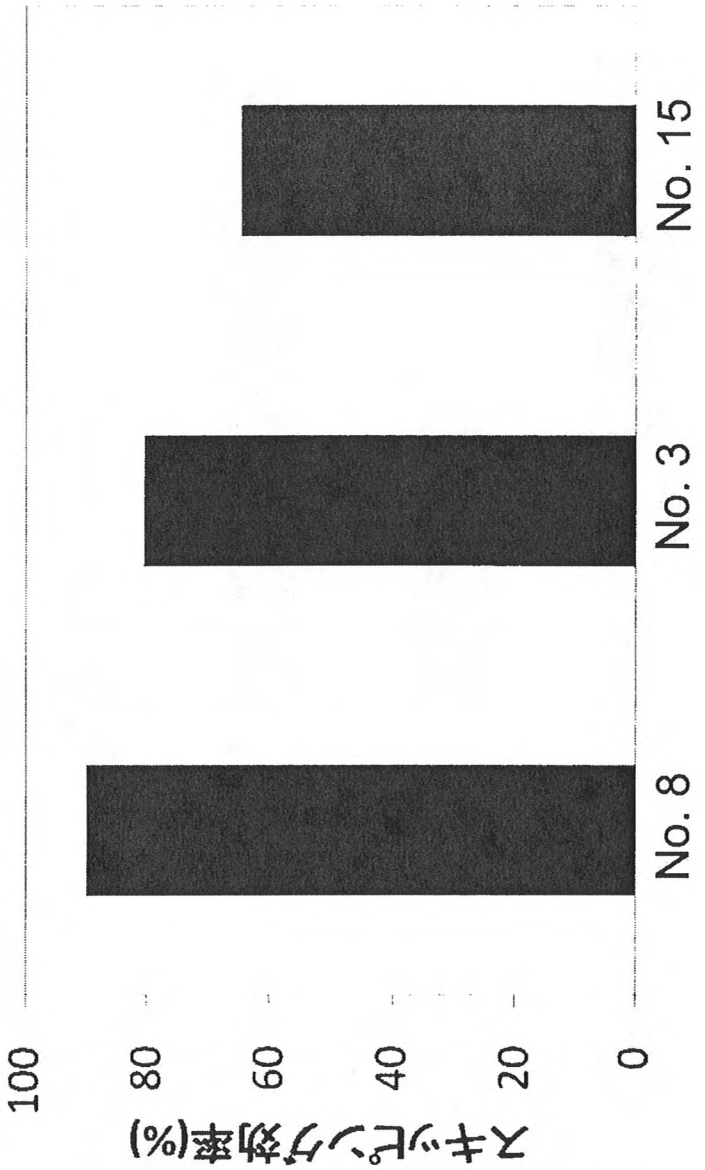
[y-axis: Skipping efficiency (%)]

Figure 3



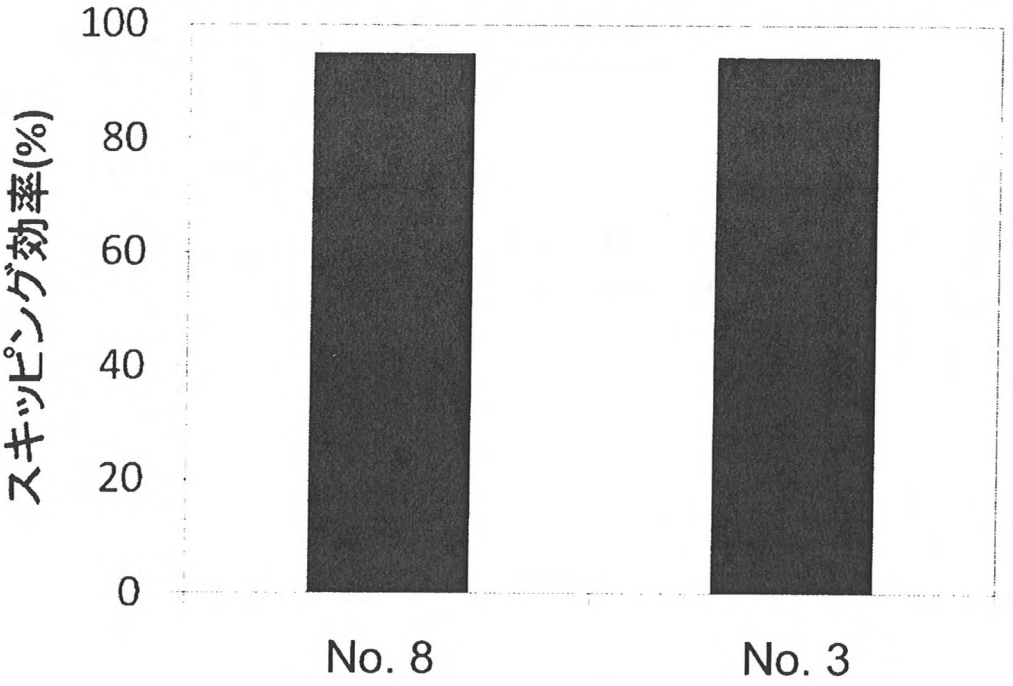
[y-axis: Skipping efficiency]

Figure 4



[y-axis: Skipping efficiency] (%)

Figure 5



[y-axis: Skipping efficiency]

Figure 6

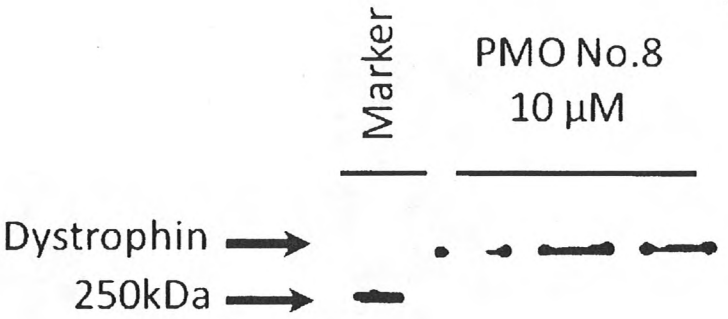
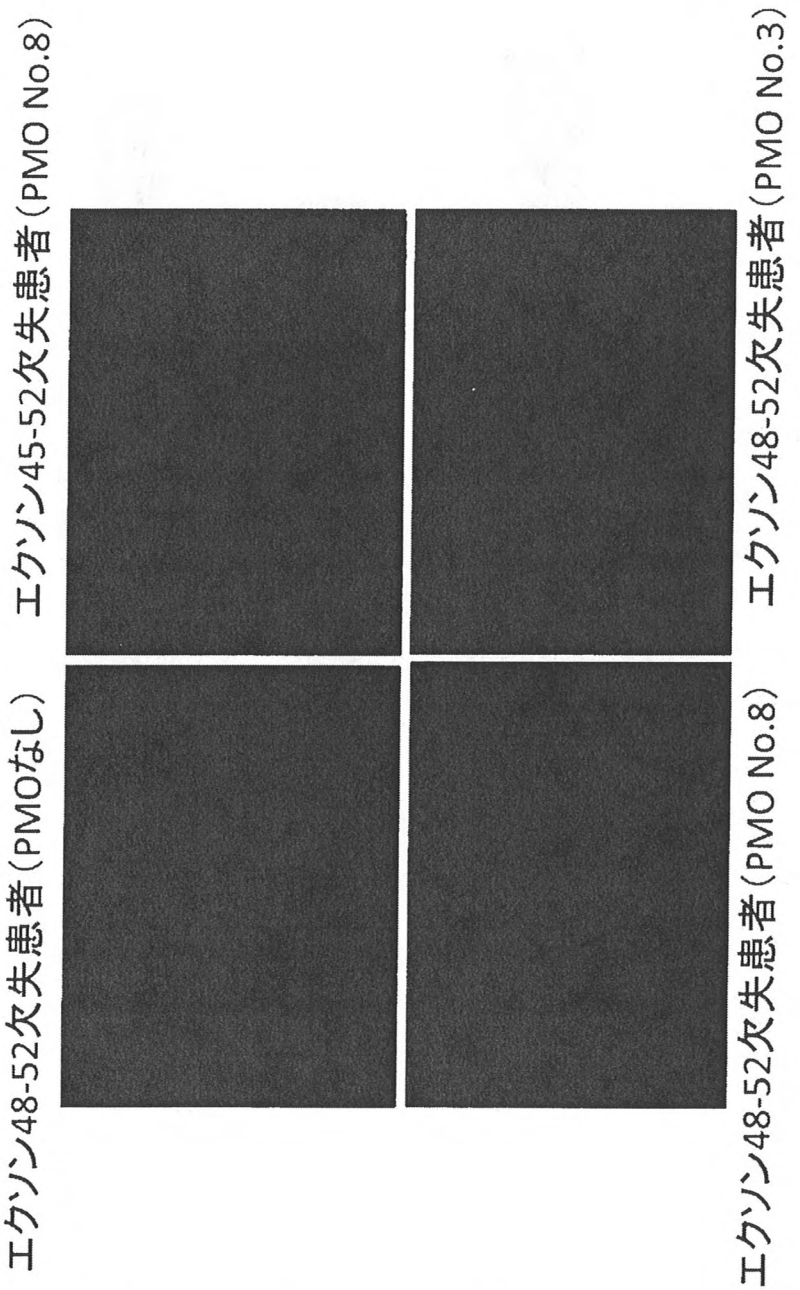
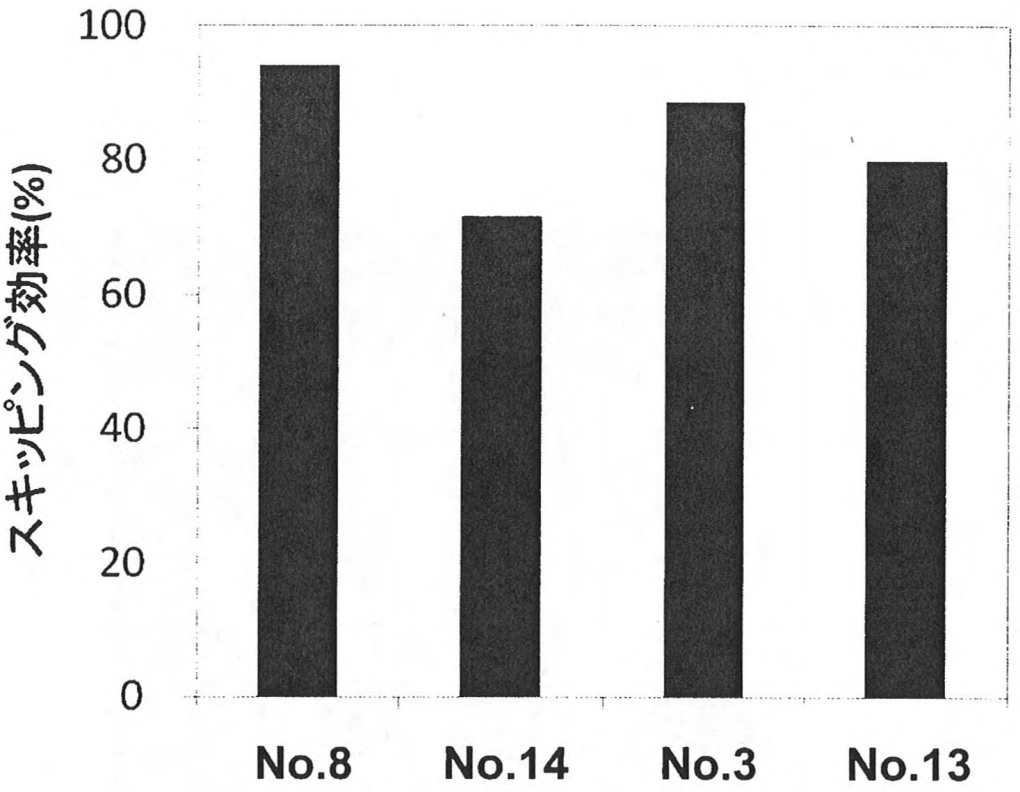


Figure 7



[Top: Exon 48-52 deletion patient no PMO, Exon 45-52 deletion patient PMO No. 8;
Bottom: Exon 48-52 deletion patient PMO No. 8, Exon 48-52 deletion patient PMO No. 3]

Figure 8



[y-axis: Skipping efficiency (%)]

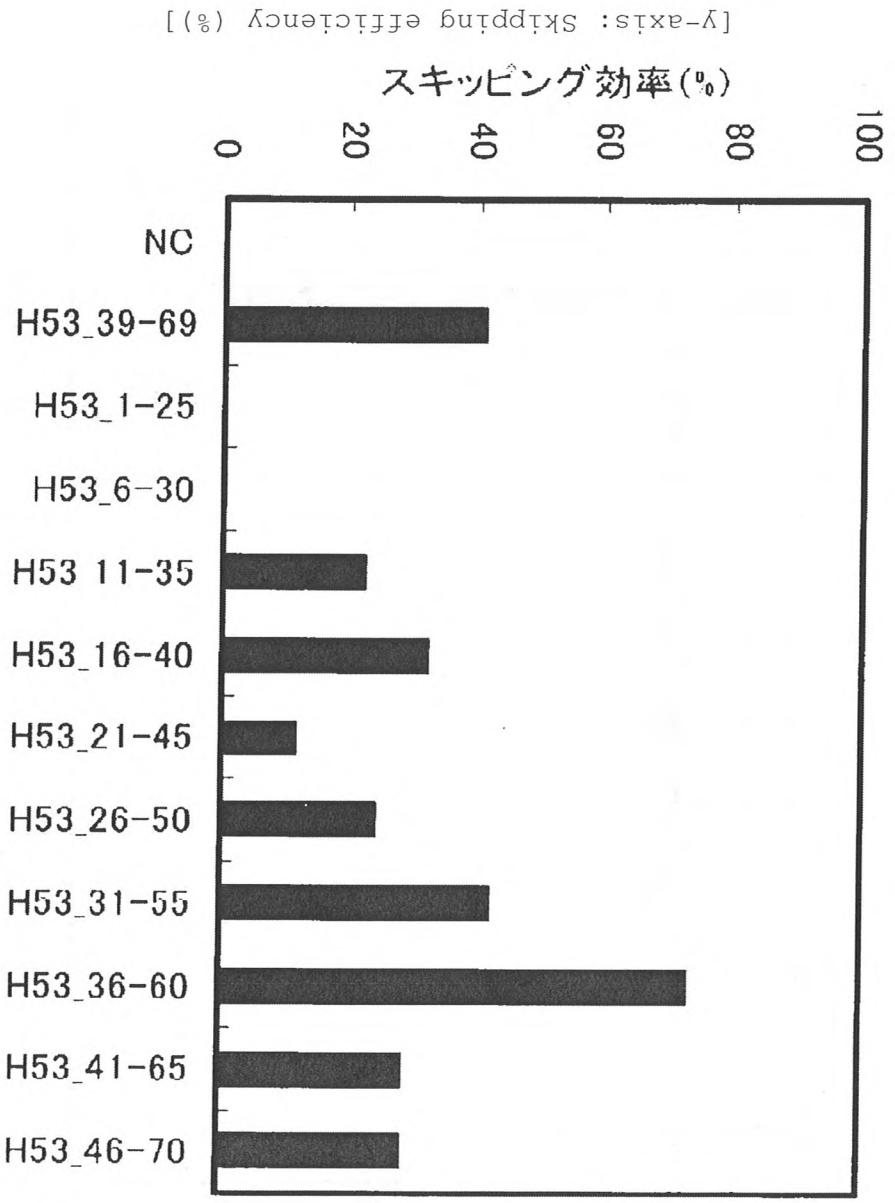
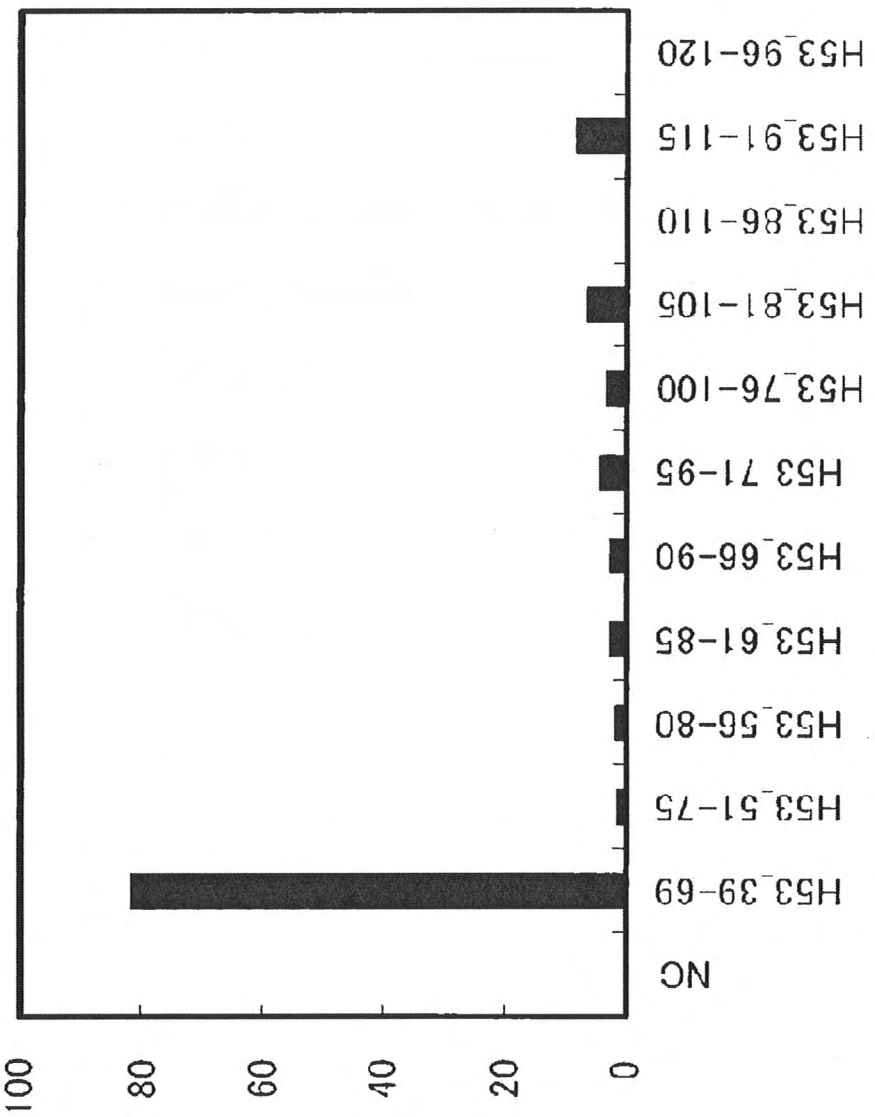
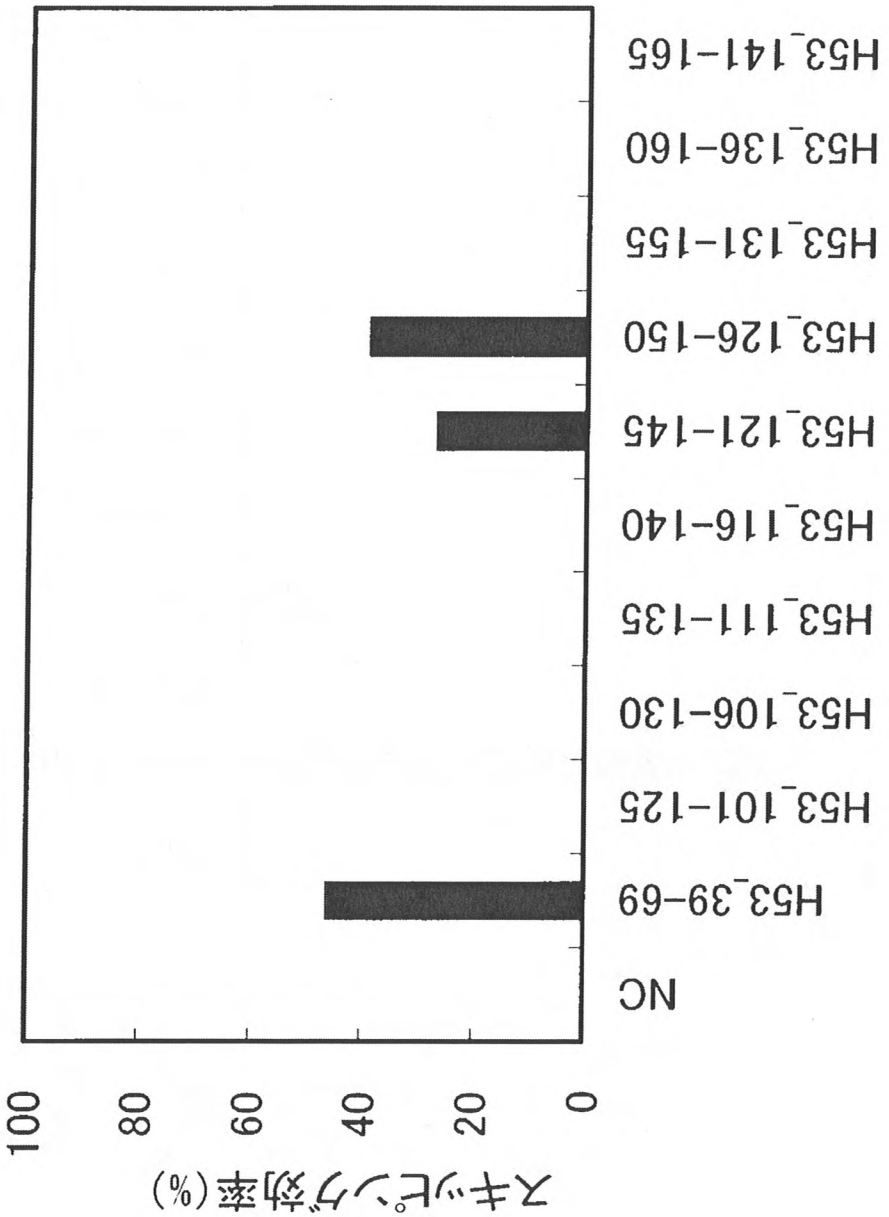

[y-axis: Skipping efficiency (%)]
スキッピング効率(%)
0
20
40
60
80
100
NC
H53_39-69
H53_1-25
H53_6-30
H53 11-35
H53_16-40
H53_21-45
H53_26-50
H53_31-55
H53_36-60
H53_41-65
H53_46-70

Figure 10



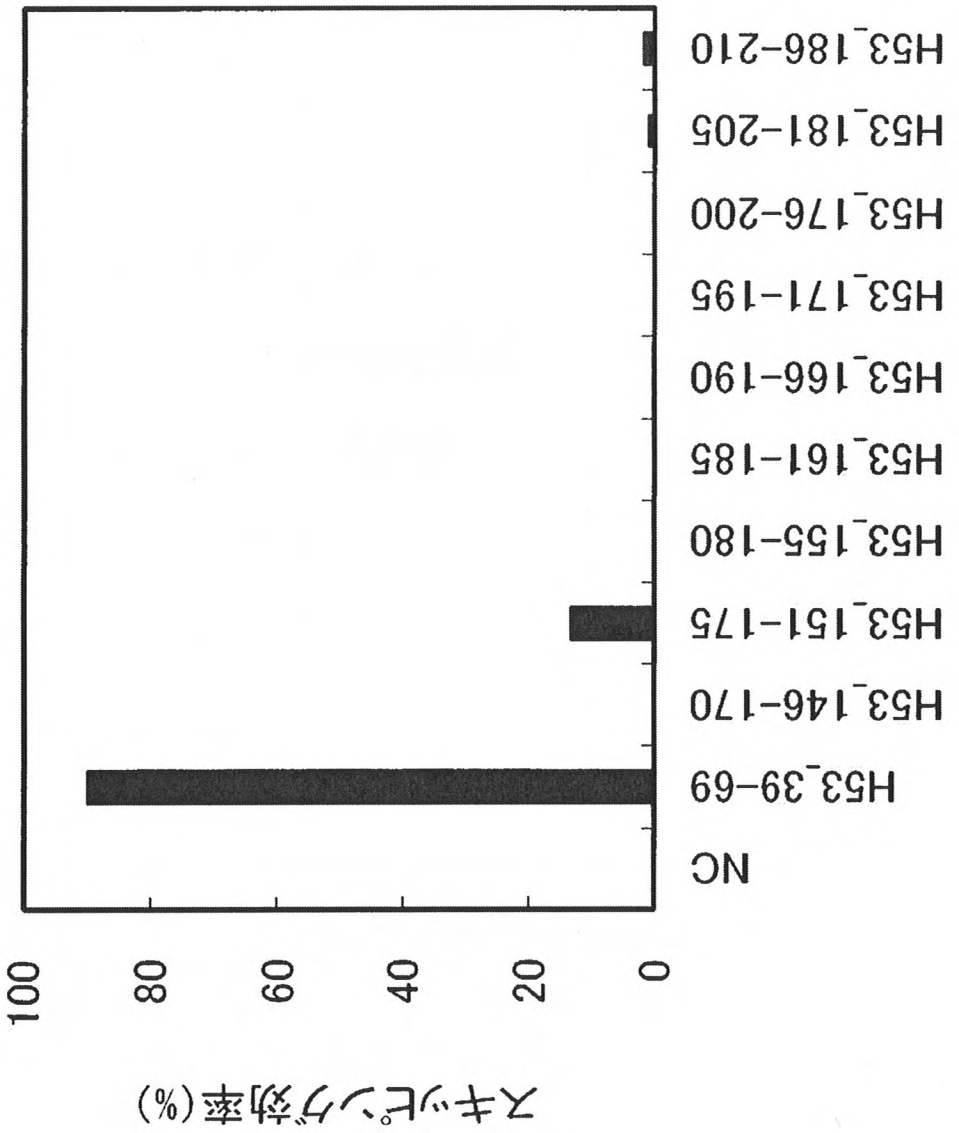
[y-axis: Skipping efficiency (%)]

Figure 11



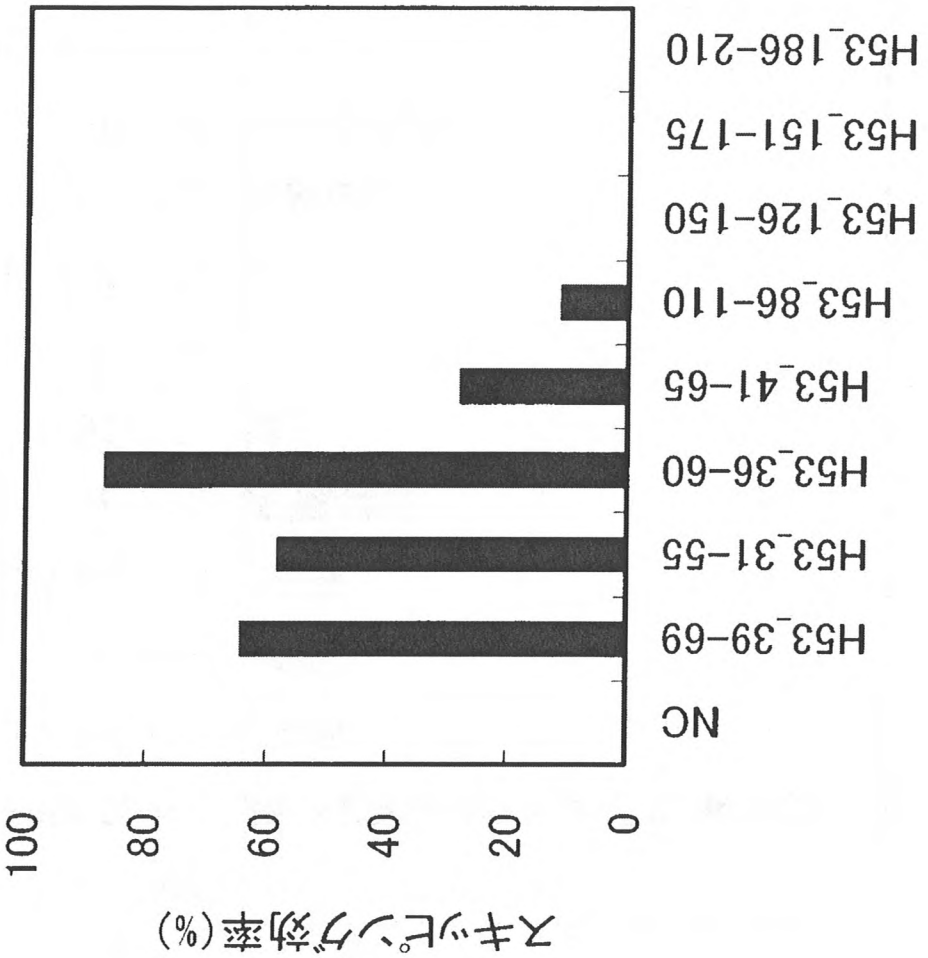
[y-axis: Skipping efficiency (%)]

Figure 12



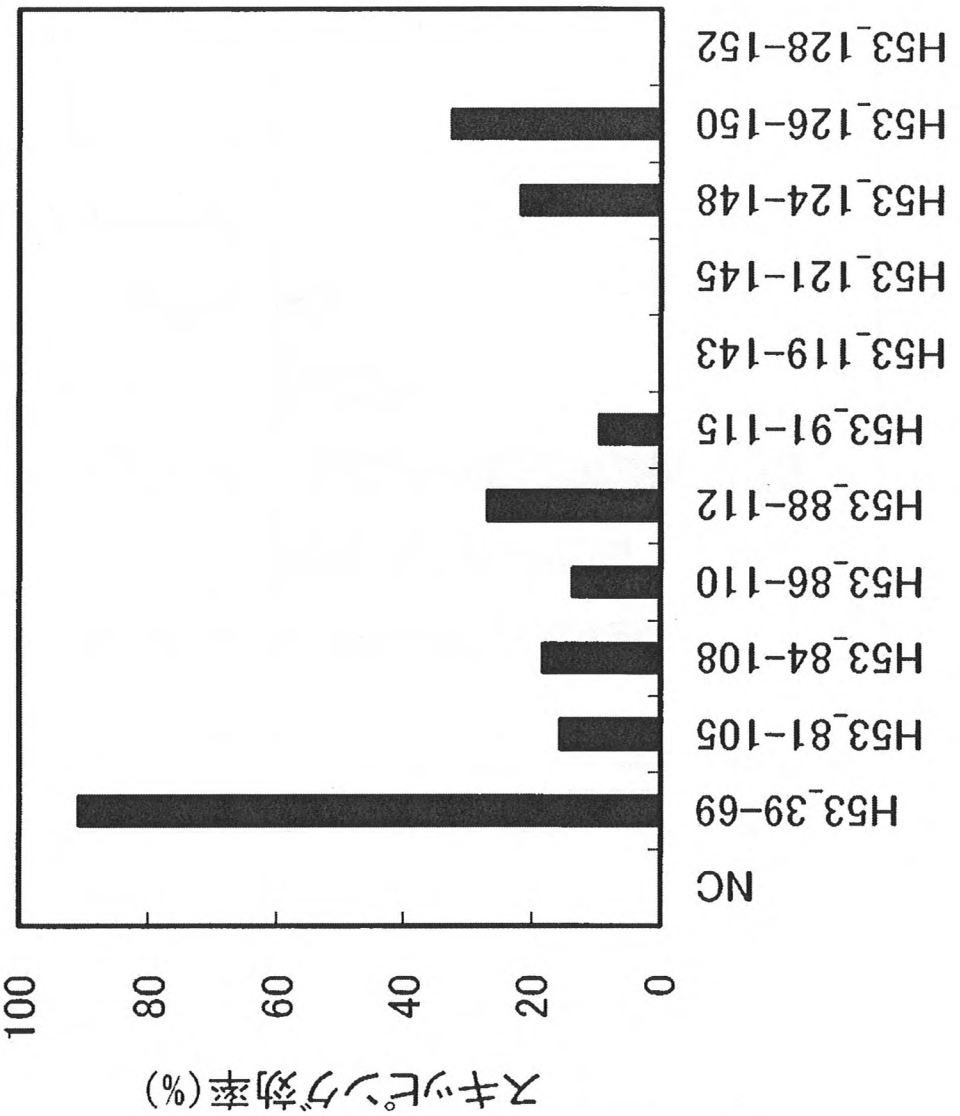
[y-axis: Skipping efficiency (%)]

Figure 13



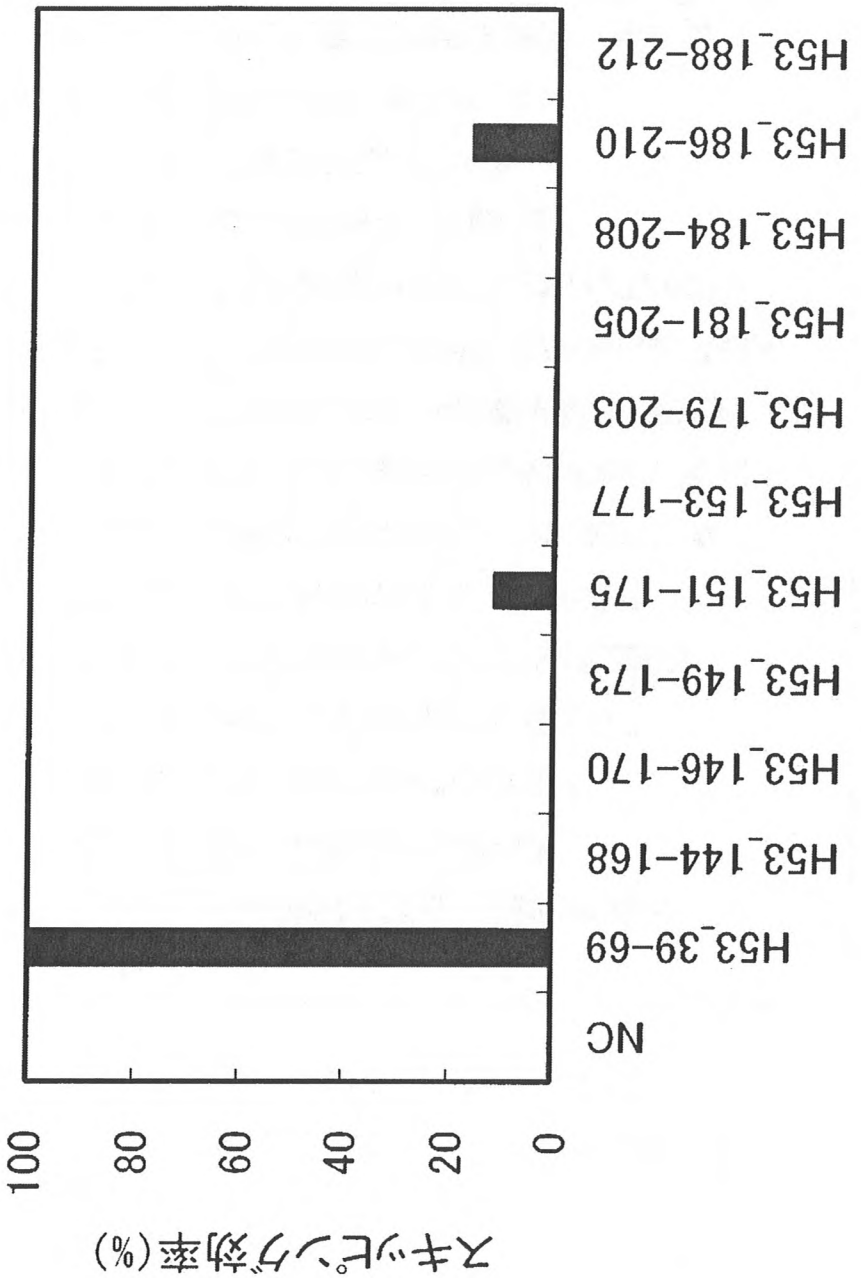
[y-axis: Skipping efficiency (%)]

Figure 14



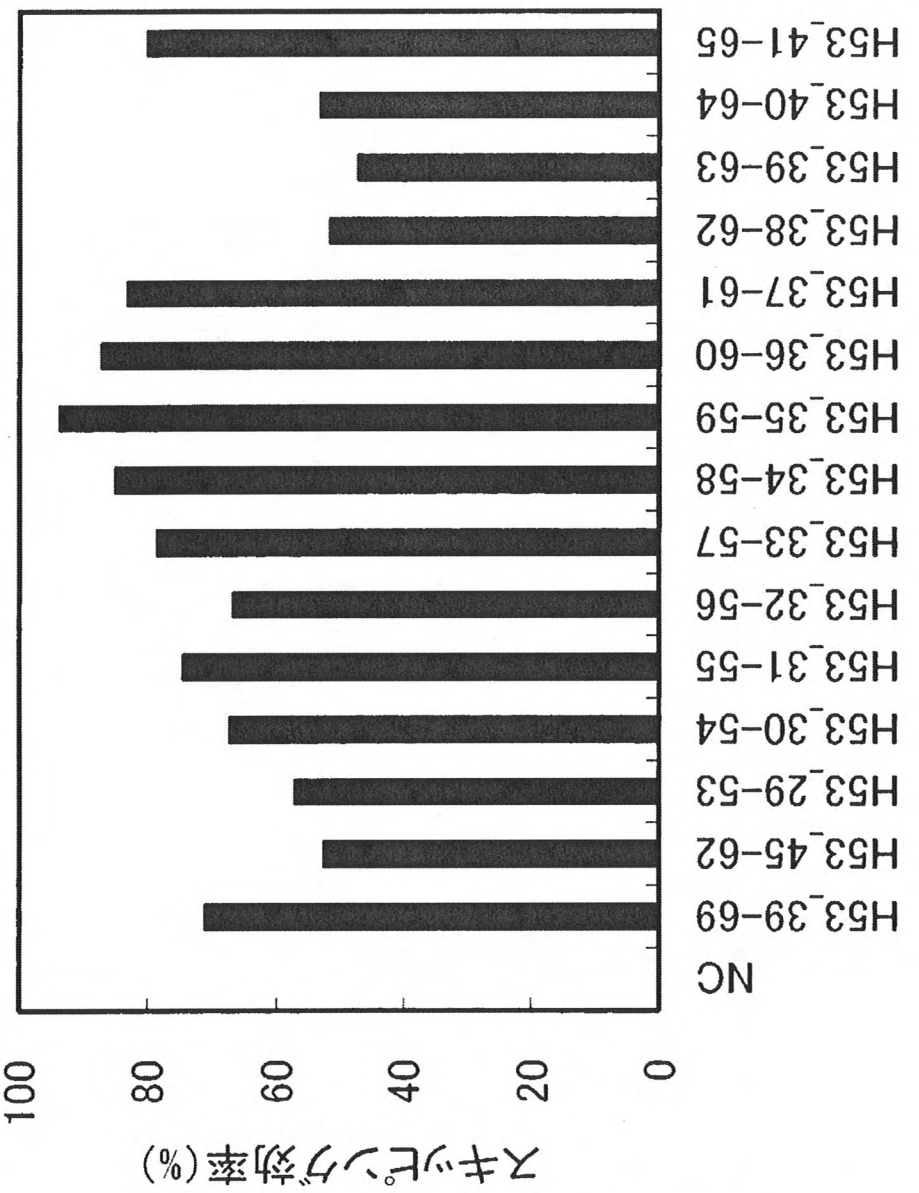
[y-axis: Skipping efficiency (%)]

Figure 15



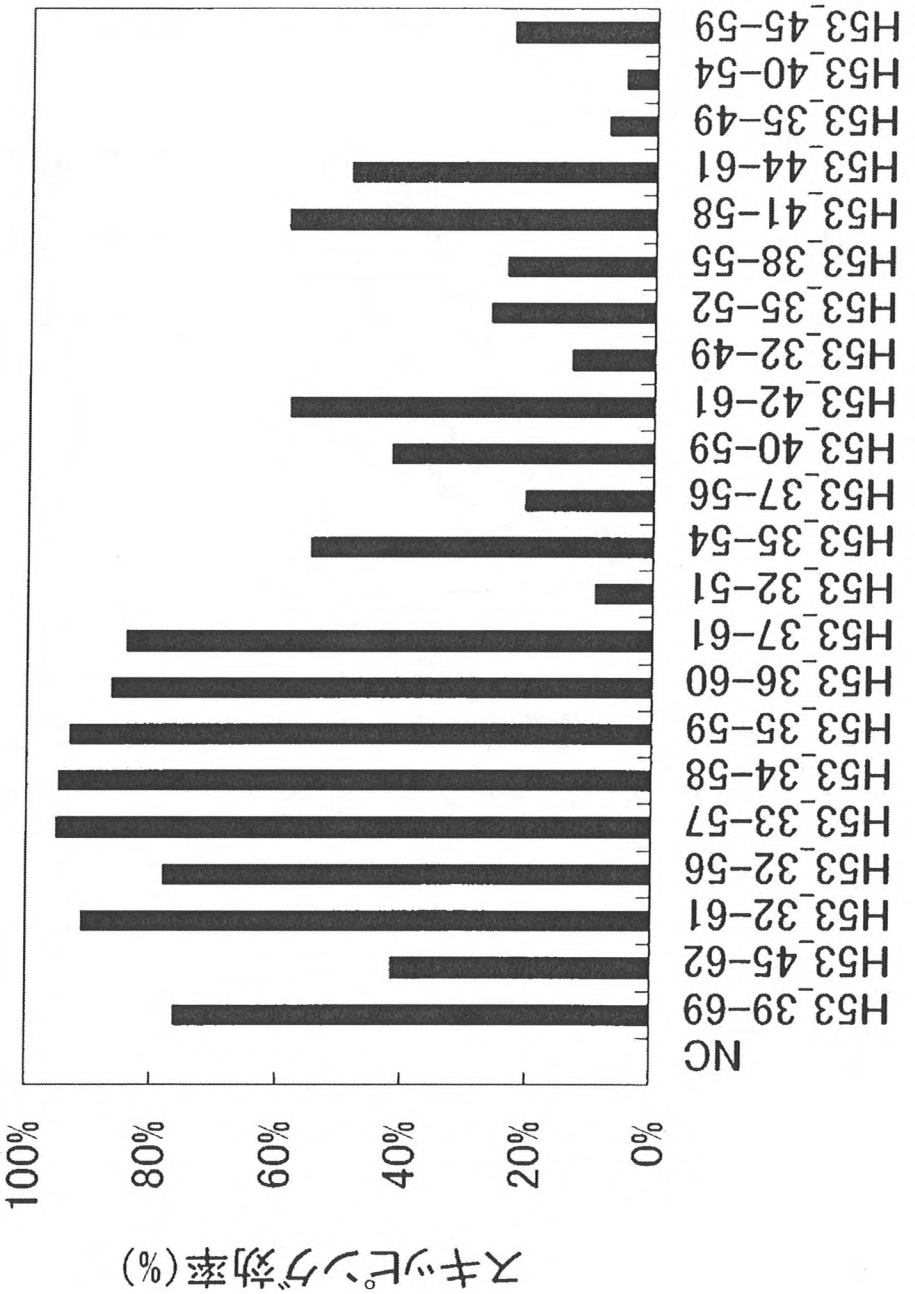
[y-axis: Skipping efficiency (%)]

Figure 16



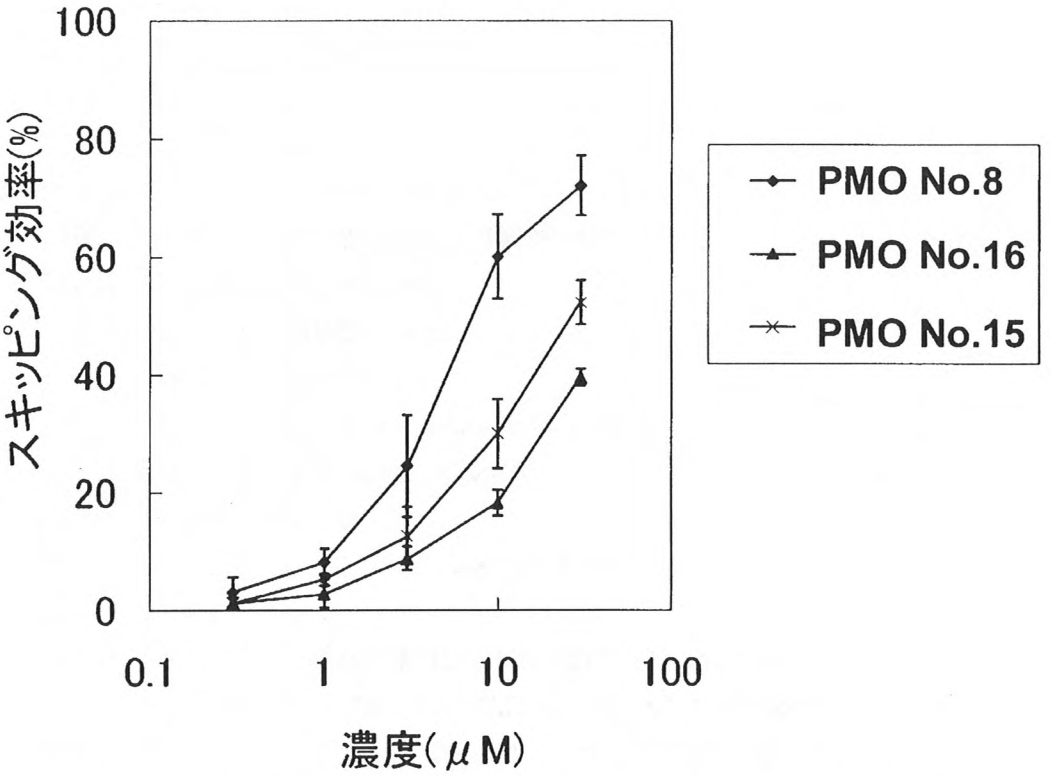
[y-axis: Skipping efficiency (%)]

Figure 17



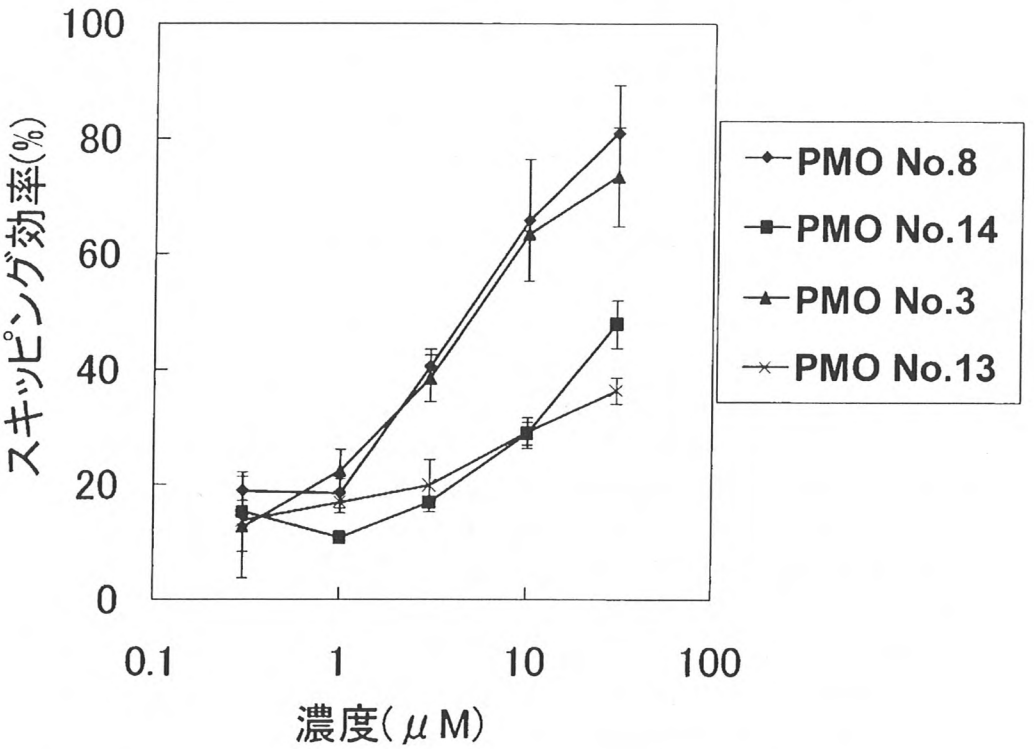
[y-axis: Skipping efficiency (%)]

Figure 18



[x-axis: Concentration (μM); y-axis: Skipping efficiency (%)]

Figure 19



[x-axis: Concentration (μM); y-axis: Skipping efficiency (%)]

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application No. :
U.S. National Serial No. :
Filed :
PCT International Application No. : PCT/JP2011/070318

VERIFICATION OF A TRANSLATION

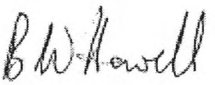
I, the below named translator, hereby declare that:

My name and post office address are as stated below;

That I am knowledgeable in the Japanese language in which the below identified international application was filed, and that, to the best of my knowledge and belief, the English translation of the international application No. PCT/JP2011/070318 is a true and complete translation of the above identified international application as filed.

I hereby declare that all the statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the patent application issued thereon.

Date: November 22, 2019



Full name of the translator : Brian William HOWELLS
For and on behalf of RWS Group Ltd

Post Office Address : Europa House, Chiltern Park,
Chiltern Hill, Chalfont St Peter,
Buckinghamshire, United Kingdom.

EXHIBIT AF

記録原本

PCT11-0042

特許協力条約に基づく国際出願願書

原本(出願用)

0	受理官庁記入欄	
0-1	国際出願番号	PCT/JP2011/070318
0-2	国際出願日	31.08.2011
0-3	(受付印)	PCT International Application 日本国特許庁

0-4	様式 PCT/RO/101 この特許協力条約に基づく国際出願願書は、	
0-4-1	右記によって作成された。	PCT-SAFE [EASY mode] Version 3.51.043.219 MT/FOP 20091001/0.20.5.17
0-5	申立て 出願人は、この国際出願が特許協力条約に従って処理されることを請求する。	
0-6	出願人によって指定された受理官庁	日本国特許庁 (RO/JP)
0-7	出願人又は代理人の書類記号	PCT11-0042
I	発明の名称	アンチセンス核酸
II	出願人	
II-1	この欄に記載した者は	出願人である (applicant only)
II-2	右の指定国についての出願人である。	米国を除く全ての指定国 (all designated States except US)
II-4ja	名称	日本新薬株式会社
II-4en	Name:	NIPPON SHINYAKU CO., LTD.
II-5ja	あて名	6018550 日本国 京都府京都市南区吉祥院西ノ庄門口町１４番地
II-5en	Address:	14, Kisshoin Nishinosho Monguchicho, Minami-ku, Kyoto-shi, Kyoto 6018550 Japan
II-6	国籍(国名)	日本国 JP
II-7	住所(国名)	日本国 JP

PCT11-0042

特許協力条約に基づく国際出願願書

原本(出願用)

III-1	その他の出願人又は発明者	
III-1-1	この欄に記載した者は	出願人である (applicant only)
III-1-2	右の指定国についての出願人である。	米国を除く全ての指定国 (all designated States except US)
III-1-4ja	名称	独立行政法人国立精神・神経医療研究センター
III-1-4en	Name:	NATIONAL CENTER OF NEUROLOGY AND PSYCHIATRY
III-1-5ja	あて名	1878551 日本国 東京都小平市小川東町４丁目１番１号
III-1-5en	Address:	1-1, Ogawahigashicho 4-chome, Kodaira-shi, Tokyo 1878551 Japan
III-1-6	国籍(国名)	日本国 JP
III-1-7	住所(国名)	日本国 JP
III-2	その他の出願人又は発明者	
III-2-1	この欄に記載した者は	出願人及び発明者である (applicant and inventor)
III-2-2	右の指定国についての出願人である。	米国のみ (US only)
III-2-4ja	氏名(姓名)	渡辺直樹
III-2-4en	Name (LAST, First):	WATANABE, Naoki
III-2-5ja	あて名	3050003 日本国 茨城県つくば市桜一丁目２１－３ルヴィオＩＩ－４０２
III-2-5en	Address:	Room 402, Ruvioll, 21-3, Sakura 1-chome, Tsukuba-shi, Ibaraki 3050003 Japan
III-2-6	国籍(国名)	日本国 JP
III-2-7	住所(国名)	日本国 JP
III-3	その他の出願人又は発明者	
III-3-1	この欄に記載した者は	出願人及び発明者である (applicant and inventor)
III-3-2	右の指定国についての出願人である。	米国のみ (US only)
III-3-4ja	氏名(姓名)	佐藤洋平
III-3-4en	Name (LAST, First):	SATOU, Youhei
III-3-5ja	あて名	6650811 日本国 兵庫県宝塚市南ひばりガ丘１丁目１３番５号
III-3-5en	Address:	13-5, Minamihibarigaoka 1-chome, Takaraduka-shi, Hyogo 6650811 Japan
III-3-6	国籍(国名)	日本国 JP
III-3-7	住所(国名)	日本国 JP

PCT11-0042

特許協力条約に基づく国際出願願書

原本(出願用)

III-4	その他の出願人又は発明者	
III-4-1	この欄に記載した者は	出願人及び発明者である (applicant and inventor)
III-4-2	右の指定国についての出願人である。	米国のみ (US only)
III-4-4ja	氏名(姓名)	武田伸一
III-4-4en	Name (LAST, First):	TAKEDA, Shin'ichi
III-4-5ja	あて名	1878551 日本国 東京都小平市小川東町４丁目１番１号独立行政法人国立精神・神経医療研究センター内
III-4-5en	Address:	c/o National Center of Neurology and Psychiatry, 1-1, Ogawahigashicho 4-chome, Kodaira-shi, Tokyo 1878551 Japan
III-4-6	国籍(国名)	日本国 JP
III-4-7	住所(国名)	日本国 JP
III-5	その他の出願人又は発明者	
III-5-1	この欄に記載した者は	出願人及び発明者である (applicant and inventor)
III-5-2	右の指定国についての出願人である。	米国のみ (US only)
III-5-4ja	氏名(姓名)	永田哲也
III-5-4en	Name (LAST, First):	NAGATA, Tetsuya
III-5-5ja	あて名	1878551 日本国 東京都小平市小川東町４丁目１番１号独立行政法人国立精神・神経医療研究センター内
III-5-5en	Address:	c/o National Center of Neurology and Psychiatry, 1-1, Ogawahigashicho 4-chome, Kodaira-shi, Tokyo 1878551 Japan
III-5-6	国籍(国名)	日本国 JP
III-5-7	住所(国名)	日本国 JP

PCT11-0042

特許協力条約に基づく国際出願願書

原本(出願用)

IV-1	代理人又は共通の代表者、通知のあて名 下記の者は国際機関において右記のごとく出願人のために行動する。	代理人 (agent)
IV-1-1ja	氏名(姓名)	小林浩
IV-1-1en	Name (LAST, First):	KOBAYASHI, Hiroshi
IV-1-2ja	あて名	1040028 日本国 東京都中央区八重洲二丁目８番７号福岡ビル９階　阿部・井窪・片山法律事務所
IV-1-2en	Address:	ABE, IKUBO & KATAYAMA, Fukuoka Bldg. 9th Fl., 8-7, Yaesu 2-chome, Chuo-ku, Tokyo 1040028 Japan
IV-1-3	電話番号	03-3273-2611
IV-1-4	ファクシミリ番号	03-3273-2034
IV-1-6	代理人登録番号	100092783
IV-2	その他の代理人	筆頭代理人と同じあて名を有する代理人 (additional agent(s) with the same address as first named agent)
IV-2-1ja	氏名	片山英二; 大森規雄; 今里崇之; 鈴木康仁
IV-2-1en	Name(s)	KATAYAMA, Eiji; OHMORI, Norio; IMAZATO, Takayuki; SUZUKI, Yasuhito
V	国の指定	
V-1	この願書を用いてされた国際出願は、規則4.9(a)に基づき、国際出願の時点で拘束される全てのPCT締約国を指定し、取得しうるあらゆる種類の保護を求め、及び該当する場合には広域と国内特許の両方を求める国際出願となる。	
VI-1	先の国内出願に基づく優先権主張	
VI-1-1	出願日	2010年 09月 01日 (01.09.2010)
VI-1-2	出願番号	2010-196032
VI-1-3	国名	日本国 JP
VI-2	優先権証明書送付の請求 上記の先の出願のうち、右記の番号のものについては、出願書類の認証謄本を作成し国際事務局へ送付することを、受理官庁に対して請求している。	VI-1
VI-3	引用による補充： 条約第11条(1)(iii)(d)若しくは(e)に規定する国際出願の要素の全部、又は規則20.5(a)に規定する明細書、請求の範囲若しくは図面の一部がこの国際出願には含まれていないが、受理官庁が条約第11条(1)(iii)に規定する要素の1つ以上を最初に受領した日において優先権を主張する先の出願にそれが完全に含まれている場合には、規則20.6に基づく確認の手続を条件として、その要素又は部分を規則20.6の規定によりこの国際出願に引用して補充することを請求する。	
VII-1	特定された国際調査機関(ISA)	日本国特許庁 (ISA/JP)

PCT11-0042

特許協力条約に基づく国際出願願書

原本(出願用)

VIII	申立て	申立て数	
VIII-1	発明者の特定に関する申立て	–	
VIII-2	出願し及び特許を与えられる国際出願日における出願人の資格に関する申立て	–	
VIII-3	先の出願の優先権を主張する国際出願日における出願人の資格に関する申立て	–	
VIII-4	発明者である旨の申立て(米国を指定国とする場合)	–	
VIII-5	不利にならない開示又は新規性喪失の例外に関する申立て	–	
IX	照合欄	用紙の枚数	添付された電子データ
IX-1	願書(申立てを含む)	6	✓
IX-2	明細書 (配列表または配列表に関連するテーブルを除く)	69	–
IX-3	請求の範囲	2	–
IX-4	要約	1	✓
IX-5	図面	19	–
IX-7a	国際出願に含まれる用紙の枚数(明細書の配列表を除く)	97	
IX-6	明細書の配列表	41	–
IX-7	合計	138	
	添付書類	添付	添付された電子データ
IX-8	手数料計算用紙	✓	–
IX-16	規則13の3に基づき提出する国際調査のための電子形式による配列表(付属書C/ST.25形式テキストファイル)	–	1 Diskette
IX-18	PCT-SAFE 電子出願	–	✓
IX-19	規則13の3に基づき提出する附属書C/ST.25形式テキストファイル内の情報が、国際出願に含まれる配列表と同一であることを確認する陳述書	✓	–
IX-19	その他	フレキシブルディスクの記録形式等の情報を記載した書面	
IX-20	要約とともに提示する図の番号		
IX-21	国際出願の使用言語名	日本語	
X-1	出願人、代理人又は代表者の記名押印		
X-1-1	氏名(姓名)	小林浩	
X-1-2	署名者の氏名		
X-1-3	権限		

PCT11-0042

特許協力条約に基づく国際出願願書

原本(出願用)

受理官庁記入欄

10-1	国際出願として提出された書類の実際の受理の日	31.08.2011
10-2	図面	
10-2-1	受理された	
10-2-2	不足図面がある	
10-3	国際出願として提出された書類を補完する書類又は図面であってその後期間内に提出されたものの実際の受理の日(訂正日)	
10-4	特許協力条約第11条(2)に基づく必要な補完の期間内の受理の日	
10-5	出願人により特定された国際調査機関	ISA/JP
10-6	調査手数料未払いにつき、国際調査機関に調査用写しを送付していない	

国際事務局記入欄

11-1	記録原本の受理の日	